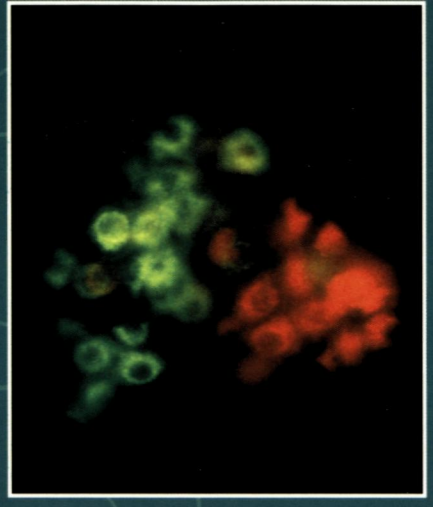
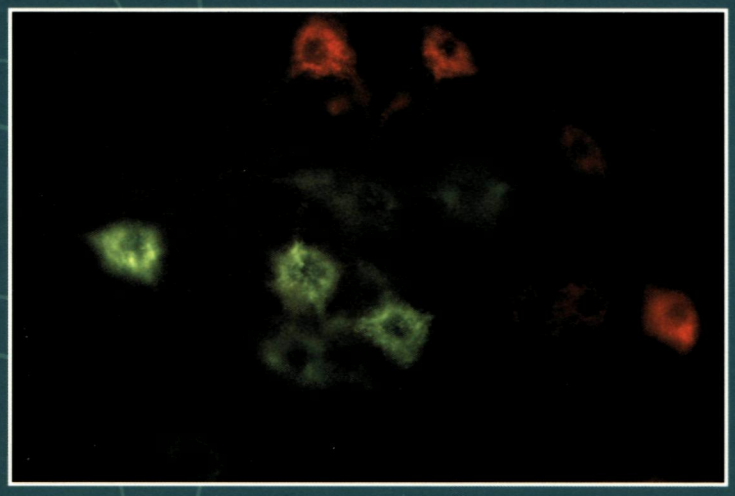


Development of the rat corticospinal tract

Target finding and fine-tuning in the cervical spinal cord



M.H.J.M. Curfs

Ter nagedachtenis
aan mijn vader

Development of the rat corticospinal tract

Target finding and fine-tuning in the cervical spinal cord

een wetenschappelijke proeve op het gebied
van de Natuurwetenschappen

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Contents

List of publications

This thesis is based on the following publications

CHAPTER 3
Curfs, M H J M , Gribnau, A A M and Dederen, P J W C , Postnatal maturation of the dendritic fields of motoneuron pools supplying flexor and extensor muscles of the distal forelimb in the rat, Development, 117 (1993) 535-541

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9 General introduction

Although in most cultures most people always have believed that a supernatural force created life, they have been fascinated by the mechanism by which new organisms are formed The theory of Aristotle (384-322 B C), who postulated that all substance including living matter was generated spontaneously from the four elements, i e air, water, earth and fire, was maintained until the middle of the seventeenth century Then, Anthonie van Leeuwenhoek (1632-1723), concluded on the basis of microscopical observation of human and canine sperm that the spermatozoon contained a complete, minuscule individual which would start to grow after being implantated into a female uterus Opposite to this theory was the idea that not the spermatozoon but rather the ovum carried the individual and that the sperm was only necessary to induce growth

Since the middle of the nineteenth century, it is accepted that all living creatures replicate through one or both of two processes asexual and sexual reproduction In the former, one or more cells separate from the organism and proliferate to form a new individual This is found exclusively in prokaryotes, plants and invertebrates When sexual reproduction is concerned, one female cell, the egg, merges with one male cell, the sperm cell, and then forms a new individual by successive divisions and the newly formation of organs and tissues This mechanism is wide-spread in nature and vertebrates even replicate exclusively through it This knowledge has led, however, to new questions how can this single, undifferentiated cell develop into an organism characterized by so many differentiated cells, tissues, and organs, each with its own different function, and which factors, genetic and environmental, play a role in this process

Before introducing the main topic of this thesis, the early embryology of mammals will be briefly discussed It should be stated however that the ontogenesis of various vertebrates shows many points of resemblance, although some differences exist After fertilization, the embryo starts dividing into 2, 4, 8, etc cells which are still undifferentiated, resulting in the morula After several more divisions, inside the embryo a cavity arises In this blastula stage the first differentiation occurs the inner cell layer or blastoderm, which will form the embryo and the outer cell layer or trophoblast which becomes part of the placenta After further proliferation the blastoderm consists of two cell layers, an outer layer or ectoderm, and an inner layer or entoderm The ectoderm eventually gives rise to the central and peripheral nervous system, epidermis including hairs and epidermal glands, sensory epithelia of eye, inner ear and nose, and enamel of the teeth, among others The entoderm eventually gives rise to the epithelia of the gastro-intestinal tract, respiratory organs and bladder, endocrine organs such as thymus, parathyroid and

thyroid, liver and pancreas, among others Along the midline the primitive streak is formed, an invagination of the ectoderm with lateral ridges The cranial end of the primitive streak is characterized by a raised node of tissue with a pit extending downward and forward beneath it From the ectoderm, cells migrate through the primitive pit to ventral and then in all directions, where they come to lie between the ecto- and endoderm, forming the mesoderm The embryo now has entered its gastrula-stage From the mesoderm, structures such as the muscles, cartilage, bones, mesenchyme, heart and blood vessels, urogenital organs, kidneys, and spleen develop

By the end of the gastrula stage, mesodermal cells migrating rostrally along the midline have formed the notochord, which in turn induces the differentiation of the above lying ectodermal cells into neuroectoderm The latter at first forms a thickening, the neural plate, but then by further proliferation and migration the plate folds and forms the neural groove Eventually, the groove closes by fusion of its lips and the neural tube is formed, from which the central nervous system originates The rostral part of the neural tube differentiates into the brain and the caudal part into the spinal cord In the anlage of the brain three vesicles arise the prosencephalon, mesencephalon, and rhombencephalon Later, five vesicles are formed by further division of the prosencephalon into telencephalon and diencephalon and rhombencephalon into metencephalon and myelencephalon, respectively From these vesicles all other brain structures are derived In certain areas nuclei will be formed by concentration of cells Each nucleus is characterized by its own specific connections with other nuclei It is the field of interest of neuroembryologists to describe and explain the formation of this organ consisting of billions of cells (in the human estimated at 1,000,000,000,000 cells) and even more numerous interconnections (each cell having a mean of approximately 1000 synaptic contacts with other cells) characterized by a high degree of complexity One research topic centers around the question of how these connections are formed and specifically, which mechanisms and molecules play a role in this process From the understanding of this process, it might be possible to recreate in adults the beneficial environmental conditions as found during embryogenesis, thus enabling functional repair of lesions in the central nervous system

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II — *Introduction and scope of the thesis*

2.1. Plasticity of the developing nervous system

Once neuroblasts have undergone their last division in the germinal layer of the neuro-ectoderm, they start to differentiate into neurons This process consists of several steps, among which migration to their final position (although not all neurons need to migrate to reach their final position), dendrogenesis, maturation of nucleus and perikaryon, axogenesis and axon elongation, target finding, synaptogenesis and fine-tuning of connections, followed by cell death of certain neurons Developing axons possess unique capabilities to grow along their substrate in order to attain their target This capacity is retained in developing axons even after part of their normal pathway has been damaged, thus resulting in at least some regeneration (e g Antonini and Stryker, 1993, Armand and Kably, 1993, Castro, 1975, Kalil and Skene, 1986, Kuang and Kalil, 1990b, Martin and Xu, 1988, Whishaw and Kolb, 1988, Xu and Martin, 1989, Yin and Oppenheim, 1992, reviewed by Kalil, 1988) In the central nervous system, this ability is lost at a certain time-point during development, resulting in irreparable loss of function after damage (e g Alstermark et al , 1981, Armand and Kably, 1993, de Ryck et al , 1992, Kalil and Skene, 1986, Kuang and Kalil, 1990b, Martin and Xu, 1988, Whishaw and Kolb, 1988, Xu and Martin, 1989, reviewed by Kalil, 1988, Schwab, 1990) In contrast, in the peripheral nervous system at least some regeneration appears to occur spontaneously (e g Borke et al , 1993, Brushart, 1993, Brushart and Mesulam, 1980, Chiu et al , 1993, Dennis and Harris, 1980, Wigston and Sanes, 1982, Wood et al , 1990) Therefore, insight in neuro-embryology yields, besides intrinsic scientific information, possibly also strategies to improve the regenerative capacity of axonal pathways and thus the repair of function of damaged nervous structures

2.2. The corticospinal tract

The corticospinal tract (CST) is one of the major fibre pathways innervating the spinal cord Although the anatomy of the brain including the concept of the cerebral cortex and the CST was still unknown, the first study describing a CST related phenomenon originated from Hippocrates (460-380 B C), who noted that after an injury of the head convulsions would seize the contralateral side of the body (see Armand, 1982) Below a short description will be given of its location in the brain and its function, based on the data in more recent studies

The CST originates from pyramidal neurons situated in layer V of the cerebral sensorimotor cortex in the telencephalon (Fig 1A) After emanating from the cortex, its axons pass through the cerebral peduncle, the pons and the medullary pyramid The latter

represents a longitudinally oriented paramedian elevation, after which the pyramidal tract is named In the medulla oblongata, most CST axons decussate to the contralateral side and extend into the spinal cord (Fig 1B) Other axons which also originate in the cortex and follow at least in part the same trajectory are the corticorubral tract, which after leaving the main trajectory in the cerebral peduncle projects to the red nucleus, the corticopontine tract, projecting to the pontine nuclei, the corticobulbar tract, which leaves the trajectory in the medulla and projects to the cranial nerve nuclei, and the corticoreticular tract projecting to the medullary reticular formation, which in turn gives rise to the reticulospinal tract (Akintunde and Buxton, 1992, reviewed in e g Armand, 1982, Kalil, 1988, Kandel and Schwartz, 1991, O'Leary and Terashima, 1988, Stanfield, 1992)

12 After entering the spinal cord, the CST in different mammalian species assumes a variable position In general, CST fibres are either crossed or uncrossed and are located either ventrally in the dorsal funiculus (e g in rodents) or in the lateral funiculus (e g in carnivores such as cat and dog, but also in primates including man) and additionally in the ventral funiculus (reviewed by Armand, 1982, Porter and Lemon, 1993) Although comparative research is far from being complete, several combinations of the above mentioned trajectories have been reported For instance in the rat and hamster, the major crossed CST component is located in the ventral part of the dorsal funiculus (Antal, 1984, Brown, 1971, Donatelle, 1977, Goodman et al, 1966, Gribnau and Dederen, 1989, Gribnau et al , 1986, Kuang and Kalil, 1990a, Liang et al , 1991, reviewed by Armand, 1982, Kalil, 1988) Additional minor components were found uncrossed in the ventral funiculus (Joosten et al , 1992, Liang et al , 1991) and crossed in the lateral funiculus and the ventral part of the dorsal horn (Joosten et al , 1991, Liang et al , 1991, Schreyer and Jones, 1982) In the cat the main crossed CST component is located differently, i e in the lateral funiculus (Alisky et al , 1992), and additional minor components are found uncrossed in the lateral funiculus and both crossed and uncrossed in the ventral funiculus (Satomi et al , 1991, reviewed by Armand, 1982)

It is generally agreed upon that the CST plays a major role in the control of fine, volitional movements of the distal part of the extremities, used for instance for grasping small morsels of food Its function can be subdivided in a sensory part, responsible for target reaching in which the somatosensory cortex is involved, and a motor part, responsible for grasping and retrieval in which the motor cortex is involved (Alstermark et al , 1981, Armand and Kably, 1993, Castro, 1972a, b, Castro Alamancos and Borrell, 1993, Flament et al , 1992, Huntley and Jones, 1991, Kuang and Kalil, 1990a, Martin, 1993, Montoya et al , 1991, Schrimsher and Reier, 1993, Wannier et al , 1991, Whishaw and Kolb, 1988, reviewed by Grillner and Dubuc, 1988, Kalil, 1988, Kuypers, 1981, 1982, Porter, 1987, Porter and Lemon, 1993) In comparing several parameters, it appeared that especially the caudal extension of the CST into the spinal cord and the deepest layer in the spinal gray (Fig 1B) with CST terminations correlated best with the ability to perform skilled finger movements (see Heffner and Masterton, 1975, Kuypers, 1981, 1982) Based on this phenomenon, mammals can be subdivided into four groups The first group of animals, among others the goat, elephant, rabbit, kangaroo, and opossum, are characterized by the presence of CST fibres only in the cervical and upper thoracic spinal cord which extend into the dorsal horn only (reviewed by Armand, 1982, Kuypers, 1981, 1982) and these animals have, if any, low digital skills (reviewed by Heffner and Masterton, 1975, Kuypers, 1981, 1982) In the second group of animals, such as cat, dog, and marmoset, CST fibres are present throughout the entire rostrocaudal extent of the spinal cord and they project into the dorsal horn and the intermediate zone (Alisky et al , 1992, Martin, 1993, Satomi et al , 1991, reviewed by Armand, 1982, Kuypers, 1981, 1982) These animals have, when compared to the first group, increased digital skills and are able to grasp small objects (Armand and Kably, 1993, reviewed by Heffner and Masterton, 1975, Kuypers, 1981, 1982) The third group of animals, such as raccoon, rat, hamster, slow loris, and rhesus monkey, has in addition to the characteristics mentioned for the second group also CST fibres projecting to the ventral horn, including the dorsolateral part of the lateral motor column (Antal, 1984, Donatelle, 1977, Goodman et al , 1966, Kuang and Kalil, 1990a, b, Liang et al , 1991, reviewed by Armand, 1982, Kalil, 1988, Kuypers, 1981, 1982) These animals have increased digital skills, e g the ability to manipulate small objects (Castro, 1972a, b, Castro-Alamancos and Borrell, 1993, Montoya et al , 1991, Whishaw and Kolb, 1988, reviewed by Heffner and Masterton, 1975, Kalil, 1988, Kuypers, 1981, 1982) Finally, the fourth group of animals, among which the chimpanzee and man, is characterized by CST innervation of the entire spinal cord, including all of the lateral motor column (reviewed by Armand, 1982, Kuypers, 1981, 1982, Porter, 1987, Porter and Lemon, 1993) In this group the highest digital skills are encountered, e g the precision grip using an opposable thumb in conjunction with the fingers (reviewed by

Heffner and Masterton, 1975, Kuypers, 1981, 1982, Porter, 1987, Porter and Lemon, 1993)

2.3. The development of the rat corticospinal tract

When studying developmental events, the accessibility to the embryo or fetus is a prerequisite Therefore, especially invertebrates and lower vertebrates, such as the roundworm *Caenorhabditis elegans*, the clawed toad *Xenopus laevis*, and the chicken, have been used extensively since their offspring, with some exceptions, develop in the external environment As a result the embryos can be monitored *in vivo* and experimental manipulations are relatively easy to perform Although these models provide much useful information, the results can not be extrapolated to mammals without great caution and additional evidence Mammalian embryos, however, develop in utero, thus complicating experimental research

The rodent CST provides a highly useful model in neuro-embryological studies, since it is the latest fibre pathway to develop and its outgrowth into the spinal cord occurs mainly postnatally In the rat, CST fibres reach the capsula interna at embryonic day 17 (E17), the pons at E18, the medulla oblongata at E19, are found past the decussation at E21 and in the cervical spinal cord segment 2 (C2) first at the day of birth (Fig 1A, de Kort et al , 1985, Gorgels, 1990, Gribnau et al , 1986, O'Leary and Terashima, 1988, Schreyer and Jones, 1982, Uozumi et al , 1988, reviewed by Stanfield, 1992) In the first postnatal week, CST fibres further extend into the spinal cord ultimately reaching sacral levels at the beginning of the second postnatal week (Donatelle, 1977, Gribnau et al , 1986, Joosten et al , 1987, 1989, 1992, Schreyer and Jones, 1982, Uozumi et al , 1988, reviewed by Stanfield, 1992)

So far, studies concerning the developing rodent CST focused on the outgrowth in the dorsal funiculus of the spinal cord It has been shown that at first few pathfinding fibres extend into the spinal cord, which are characterized by growth cones at their distal ends with complex protrusions These fibres are followed later by the bulk of axons which fasciculate upon these so-called pioneer fibres and have growth cones of a less complicated structure (de Kort et al , 1985, Gorgels, 1991a, Gorgels et al , 1989a, Joosten, 1989a, b, 1991b, Joosten et al , 1989, 1990, 1992, Schreyer and Jones, 1982, reviewed by Stanfield, 1992) The influence of several factors upon the pathfinding by the growth cones has also been investigated Among these factors are glia, such as oligodendrocytes and astrocytes (Gorgels, 1991b, Schwab and Schnell, 1991, reviewed by Schwab, 1990), trophic factors produced by astrocytes, e g GFAP and vimentin (Joosten, 1989b), growth associated proteins such as B-50 also known as GAP-43 (Gorgels et al , 1987, 1989b, Kalil and Skene, 1986, reviewed by Kalil, 1988), the neural cell adhesion molecule L1 (Joosten, 1990, 1991a, Joosten and Gribnau, 1989, Joosten et al , 1990), and the nerve terminal protein NT75 (Cabalka et al , 1990) The process of CST outgrowth is initially characterized by aspecificity, followed later by the elimination of the aberrant fibres Cortical neurons in areas projecting to the spinal cord also form aberrant collaterals projecting to the pons which are eliminated later Likewise, cortical neurons in areas projecting to the pons also transiently project to the spinal cord (Joosten et al , 1987, Joosten and van Eden, 1989, O'Donoghue et al , 1993, O'Leary and Terashima, 1988, Uozumi et al , 1988, reviewed by Kalil, 1988, O'Leary et al , 1990, Stanfield, 1992)

Two days after the arrival of CST axons at a given cervical or lumbar spinal cord segment, the fibres start their outgrowth into the spinal gray matter (Donatelle, 1977, Gribnau et al , 1986, Joosten, 1989a, Joosten et al , 1987, 1994, Schreyer and Jones, 1982, reviewed by Stanfield, 1992) This 'waiting period' is probably related to the formation of axon collaterals by interstitial sprouting (O'Leary and Terashima, 1988) It is assumed that cells in the spinal gray, i e the target, play a role in the process of target-finding, synaptogenesis and subsequent fine-tuning (Joosten et al , 1991, reviewed by O'Leary et al , 1990), in analogy to other developing nervous structures, such as the visual system and the motor unit (Brown and Booth, 1983, Caroni and Becker, 1992, Chiu et al , 1993, Lohof et al , 1993, Vanselow et al , 1990, reviewed by Goodman and Shatz, 1993, Kalb and Hockfield, 1992, Lichtman and Balice-Gordon, 1990, Lowrie and Vrbova, 1992, Navarette and Vrbova, 1993, Oppenheim, 1989)

2.4. Scope of this thesis

Knowledge of the target of the CST and the influence of that target on the process of outgrowth and the establishment of correct synaptic connections is sparse This thesis therefore focuses on the following questions

- What is the target of the CST in the rat cervical spinal cord?
- What is the influence of this target on the outgrowing CST?

Since the CST is especially involved in digital flexion, in chapter 3, the maturation of motoneurons inner-

vating a flexor muscle in the distal forelimb is described, with special emphasis on their dendrites These motoneurons were retrogradely labelled with the non-toxic subunit B of cholera-toxin As a reference, motoneurons innervating an extensor muscle in the distal forelimb, involved in locomotion, were also labelled An increase, followed by a progressive reduction of the dendritic field was found in both motoneuron populations

Chapter 4 describes the outgrowth of the CST into the cervical spinal gray using anterograde tract-tracing with horseradish peroxidase This process is not yet investigated in detail in the rat and the scarce pertinent data in literature show a large discrepancy It was found that corticospinal fibres progressively extend into the spinal gray, then overgrow their target, and that finally redundant fibres are subsequently eliminated

14 Corticospinal fibres were found to overlap with flexor motoneuron dendrites (chapters 3 and 4) Hence, in chapter 5 the question as to whether direct corticospinal-motoneuronal synaptic contacts exist and on what postnatal age these are formed was tackled by using a double-labellings technique the CST was labelled anterogradely with horseradish peroxidase and the motoneurons retrogradely with cholera toxin subunit B Double labelled synaptic contacts were found at the ultrastructural level in the adult rat, thus confirming that flexor motoneurons are a direct target for the CST It was further established that these synapses are first formed between postnatal day 5 and 7

Since the CST exerts its influence not only directly on motoneurons, but also indirectly via interneurons, in chapter 6, the interneurons innervating the distal forelimb flexor motoneurons were retrogradely labelled with the transneuronally transporting pseudorabies virus Several labelled populations of interneurons were found, which showed consistency at all postnatal ages investigated

In chapter 7, the cervical spinal interneurons upon which the CST projects were investigated anterogradely using the transneuronal expression of the immediate-early gene c-fos, which is induced after a neuron is stimulated The cerebral sensorimotor cortex was stimulated using kainate, a potent glutamate agonist In section 7 1 the labelled interneuron populations in the cervical spinal cord of adult rats were compared to the field of CST labelling with HRP Several populations of interneurons were encountered and it appeared that one subpopulation correlated particularly well with the CST terminal field Section 7 2 describes the interneuron populations in rats at postnatal day 14 and in adult rats labelled with c-fos after kainate stimulation of the cerebral motor cortex As based on the decreased number of interneurons in the adult when compared to postnatal day 14, it is concluded that transient functional contacts are formed during development between the CST and its target

Finally, the results obtained in this thesis are discussed and summarized in chapter 8

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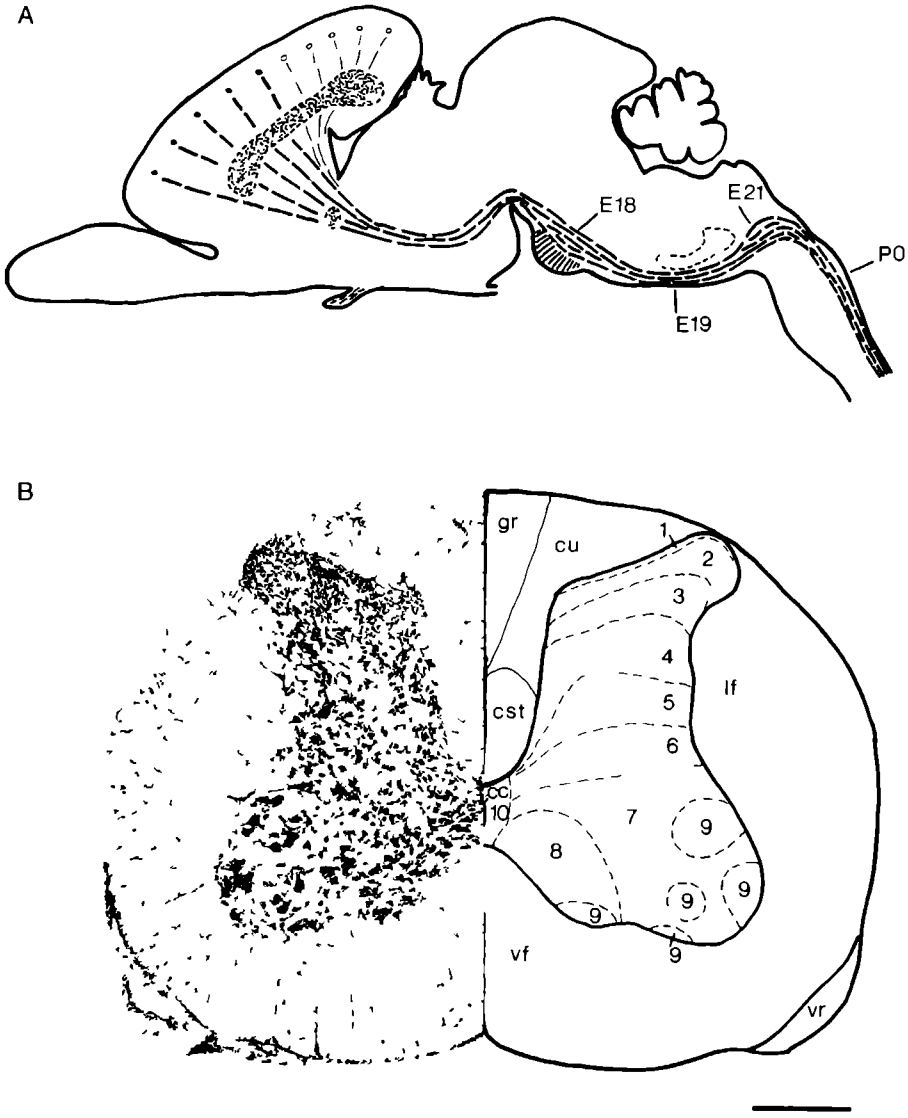

Fig 1 A Schematized drawing of the brain of a rat at P4 showing the course of the CST in the brain and its position at several ages The site of origin is indicated by the circles and the course by the dashed lines It should be noted that at this age, pyramidal neurons throughout the entire cortex project to the spinal cord Transient projections, i e the fibres which are eliminated during development, are indicated by the thin dashed line At embryonic day 18 (E18) CST fibres have reached the pons, at E19 the medulla oblongata, at E21 the decussation where the CST axons cross to the contralateral side, and at the day of birth (P0) the fibres have entered the upper cervical spinal cord segment 5 B Combined illustration of the adult rat cervical spinal cord Left half photomicrograph of a cresyl violet stain (after Paxinos and Watson, 1982) and right half schematized drawing of the section in the left half, depicting the structures encountered in this thesis 1-10 = spinal grav laminae 1-10, cc = central canal, cst = corticospinal tract, cu = fasciculus cuneatus, gr = fasciculus gracilis, lf = lateral funiculus, vf = ventral funiculus, vr = ventral root Scale bar = 0,5 mm

19 Postnatal maturation of the dendritic fields of motoneuron pools supplying flexor and extensor muscles of the distal forelimb in the rat

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Summary

In the rat cervical spinal cord the corticospinal projection on motoneurons either direct or indirect (via interneurons) comes about postnatally making it accessible for experimental research Therefore, the postnatal developmental changes of motoneurons and in particular their dendritic fields were examined Motoneurons innervating the two antagonistic muscles in the distal forepaw, the m flexor digitorum profundus and the m extensor digitorum communis, were retrogradely labelled by intramuscular injections of cholera toxin subunit B conjugated with horseradish peroxidase in rats of various postnatal ages Following a 48-72 hour survival period the motoneurons and their dendritic fields were studied in the seventh and eighth cervical spinal cord segments

Both the number and the position of motoneurons were found to remain constant throughout postnatal development Extensor motoneurons were positioned dorsolaterally in the ventral horn at the border of grey and white matter, flexor motoneurons were in general medial to extensor motoneurons The results on the dendritic field demonstrate firstly, that during postnatal development the extension of the dendrites

of both flexor and extensor motoneurons changes from spreading out in all directions at postnatal day 2 to spreading in only a few, specific directions from postnatal day 21 onwards, with the restriction that both motoneuron pools follow a different time scale to achieve this. Secondly, both pools have a temporal dendritic component extending into the white matter of the lateral funiculus. Thirdly, the dendritic extension pattern of flexor motoneurons differs from that of extensor motoneurons. the former has a permanent component in the medial part of lamina VI while the latter only has a transient component (from postnatal day 2 to 10) in the lateral part of lamina VI. The functional implications of the different dendritic extension patterns are discussed.

Introduction

20 The rat corticospinal tract (CST) is an important descending pathway from the cortex to the spinal cord and plays a role in the control of voluntary movements through contacts with motoneurons either directly (Liang et al , 1991) or through interneurons. Lesions of the CST result in the fine digital flexion movements being affected (Castro, 1972). Understanding of the development (e.g. outgrowth, target finding, synaptogenesis) of the CST might provide insight into how functional repair can be achieved after damage to the central nervous system CST outgrowth throughout the spinal cord occurs postnatally (e.g. Schreyer and Jones, 1982; Gribnau et al., 1986) making it accessible for experimental research.

In order to study the synaptogenesis of the rat CST either directly or indirectly with motoneurons (MNs), we first examined the developmental changes in MNs projecting to the lower forepaw with particular attention to their dendritic patterns, comprising most of their receptive fields. Two muscles in the distal forepaw were under investigation: the m. flexor digitorum profundus and its functional antagonist the m. extensor digitorum communis. Since in younger rats, the muscles are not as well differentiated as in the older stages these muscles will be more generally referred to as flexor- (FLEX) or extensor-muscles (EXT) respectively.

The position of MNs in several parts of the spinal cord has received much attention using various species and different techniques (Goering, 1928; Reed, 1940; Romanes, 1951; Sterling and Kuypers, 1967, Cruce, 1974; Baulac and Meiniger, 1979; Fritz et al , 1981, 1982, 1986a, 1986b; Jenny and Inukai, 1983; Mutai et al., 1986; Oka et al., 1989, Scarisbrick et al., 1990). It is generally accepted that MNs supplying different muscles are found in separate longitudinal columns (the so-called motor pools; Romanes, 1964), their position depending upon the location of the muscles they project to, i e. MNs projecting to rostral or, in the case of the limb, proximal musculature are, in general, found in a more rostral position than MNs projecting to caudal or distal musculature In the transverse plane, MNs projecting to functionally or ontogenetically related muscles occupy a relatively constant position (e.g. FLEX-MNs in general medial to EXT-MNs).

The position of the FLEX- and EXT-MNs and their dendritic fields in the rat cervical spinal cord have, so far, not been studied. Application of HRP to the cut radial or median nerves that supply, amongst other muscles, EXT and FLEX, respectively, revealed that MNs projecting through the radial nerve are located from the fifth cervical to the first thoracic spinal cord segment in the dorsolateral part of the ventral horn, at the border of grey and white matter. The MNs with their axons in the median nerve are located from the sixth cervical to the first thoracic and also in the dorsolateral part of the ventral horn but in general medial to radial nerve MNs (Baulac and Meininger, 1979; Scarisbrick et al , 1990)

A major disadvantage of the HRP-technique is that the dendritic arbour of a MN is not completely revealed. In the last decade another tracer has been shown to be superior to HRP, which is passively taken up by axon terminals: the enterotoxin cholera-toxin produced by *Vibrio cholerae* (CT, Trojanowski et al , 1982; Wan et al., 1982). CT and its non-toxic subunit B (CTB) are actively taken up. Other advantages of CT or CTB are the complete filling of dendritic structures in a Golgi-like way and the simple detection when conjugated with HRP (CT-HRP. Goldstein et al., 1990, CTB-HRP Beattie et al , 1990, Mong, 1990; Liang et al., 1991).

The present study reports on the postnatal development of the dendritic fields of MNs projecting to EXT- and FLEX-muscles in the rat cervical spinal cord using small injections of CTB-HRP Part of these results were published in abstract form elsewhere (Curfs et al , 1992).

Materials and methods

Animals

Postnatal Wistar rats (Central Animal Laboratory, University of Nijmegen) of either sex were used, ranging in age from postnatal day 0 (P0) to young adult (P60); the day of birth was designated P0. At least 4 animals per age group were examined; the ages of the animals given in this paper are the ages at their respective days of killing

Labelling of MNs using CTB-HRP

After anaesthetizing the animals with sodium pentobarbital (depending on age 18-60 mg per kg body weight, i p), the skin of the forepaw was incised dorsally or ventrally to expose the right EXT- or the left FLEX-muscles respectively, care was taken to avoid damaging the muscle fascies (damage results in extensive diffusion of tracers, Haase and Hrycyshyn, 1986) Using a 5 µl Hamilton syringe fitted with a glass micropipette, 0 5 µl of a 0 1% CTB-HRP solution (List Biological Laboratories, Inc) was pressure injected into the EXT- or FLEX-muscles Postinjection survival times were kept constant at 48 hours, except for the P60-animals in which 72 hours yielded optimal results The animals were reanaesthetized (sodium pentobarbital, 25-90 mg per kg body weight, i p) and transcardially perfused with ice-cold 5% sucrose in 0 1 M phosphate buffer (PB, pH 7 2), followed by an ice-cold mixture of 1% paraformaldehyde and 2% glutaraldehyde in the same buffer After perfusion, the brain and spinal cord were dissected from the skull and spine respectively, postfixed by immersion for 2 hours in the fixative mentioned above, cryoprotected by immersion overnight in 0 1 M PB containing 20% sucrose and embedded in 15% gelatin in the same solution The material was cut on a freezing microtome into 30 µm sections either in the horizontal or the transverse plane All horizontal and alternate transverse sections were reacted for HRP histochemistry using tetramethylbenzidine (TMB) as a chromogen (Mesulam, 1978), mounted onto glass slides, counterstained with neutral red, dehydrated and coverslipped with Depex

Drawings of the labelling in the seventh and eighth cervical spinal cord segments (pilot studies revealed that FLEX- and EXT-MNs were located in these two segments) were made under dark-field illumination using a Zeiss microscope equipped with a drawing tube Each segment was divided into three parts a rostral, middle and caudal third and every transverse section was projected onto its respective third The resulting composite illustration was equally comparable in all animals studied Two sets of composite illustrations were drawn one to show the position of the MNs and one to show the dendritic field Photomicrographs were taken using an automatic Zeiss-photomicroscope II

Results

CTB-HRP proved to be very suitable for retrograde labelling of MNs since cell bodies were densely filled and cell processes showed granular filling with the TMB-reaction product, dendrites could often be followed up to about 0 5 mm (Fig 1) Since the main scope of the present study concerned the development of the dendritic field and in particular the radially extending dendrites, only results obtained in transverse sections will be described It should be mentioned, however, that these data are in accordance with those obtained from horizontal sections

Position of MNs

MNs projecting to the EXT- or FLEX-muscles were each found in a separate longitudinal column

Throughout postnatal development, labelled MNs of both muscles maintained the same position in the spinal cord (relative to landmarks such as the central canal and the lateral funiculus, as well as to each other) in both the dorsoventral and the lateromedial axis Since the spinal cord shows growth in all dimensions, the MNs come to lie scattered in their respective columns EXT-MNs were located in the dorsolateral part of the ventral horn in lamina IX of the grey matter, at the border of spinal white and grey throughout the seventh (C7) and eighth (C8) cervical spinal cord segments (Figs 2, 3) FLEX-MNs were also located in the dorsolateral part of lamina IX, though generally medial to EXT-MNs (Figs 2, 3) This can also clearly be seen in the horizontal sections (Fig 1B)

Development of the MN dendritic field

The extension pattern of MN dendrites in the grey matter is described according to the laminar scheme of the rat cervical spinal cord presented by Molander et al (1989)

EXT-MNs

POSTNATAL DAY 2 (P2, Fig 4) Dendrites of MNs labelled after injections of CTB-HRP were found throughout C7-C8 Dendrites stretched out radially in all directions, dorsally, dorsolaterally, laterally and ventrolaterally into the lateral funiculus, dorsomedially into the lateral part of lamina VI and medially, ventromedially and ventrally into lamina VII (it should be mentioned however that axons cannot be distinguished from dendrites)

POSTNATAL DAY 4 (P4) In comparison with P2 there was an increase both in the amount of label and in dendritic extension The increase in dendritic extension was particularly due to those dendrites located in the grey matter extending medially and ventromedially further into lamina VII

POSTNATAL DAY 7 (P7, Fig 5) An increase in the amount of label was observed Compared to P4 the dendritic extension pattern remained unchanged

POSTNATAL DAY 10 (P10) When compared to P7 a decrease in the amount of label was observed At this

age virtually no dendrites were observed extending laterally and ventrolaterally into the white matter, in all other directions the loss of dendrites was approximately equal

POSTNATAL DAY 14 (P14) The amount of label continued to decrease Dendrites extending dorsolaterally and dorsally into the lateral funiculus almost completely disappeared, as a result virtually no dendrites reached out into the white matter at this age In addition, no labelled dendrites were found in lamina VI, consequently almost all labelled dendrites were confined to lamina VII

POSTNATAL DAY 21 (P21, Fig 6) The amount of label further decreased, in particular in the medial and ventromedial directions No other changes were observed when compared to P14

POSTNATAL DAY 60 (P60) No changes in relation to P21 were observed Labelled dendrites were found throughout C7 and C8 Dendrites extended mainly dorsomedially into the dorsal part of lamina VII, ventrally into the ventral part of lamina VII and medially into lamina VII

FLEX-MNs

POSTNATAL DAY 2 (P2, Fig 4) Dendrites of MNs labelled after injection of CTB-HRP were also found throughout C7 and C8, however they outnumbered those of the EXT-side The dendritic extension pattern was comparable to that of the EXT-MNs in that they spread out in all directions, however, they extended further, especially dorsolaterally into the lateral funiculus and dorsomedially into the medial part of lamina VI

POSTNATAL DAY 4 (P4) An increase in the amount of label in all directions and in dendritic extension was observed, especially of those spreading out into lamina VII

POSTNATAL DAY 7 (P7, Fig 5) A decrease in the amount of label was observed This decrease was observed in all directions but was mainly due to dendrites no longer extending dorsally towards and into the lateral funiculus, medially and ventromedially into lamina VII and ventrolaterally into the white matter Relative to the EXT-side less label was observed

POSTNATAL DAY 10 (P10) A decrease in the amount of label was observed, this decrease however was not as large as on the EXT-side This resulted in an equal amount of labelled dendrites on both the EXT- and the FLEX-side Nearly no dendrites were observed extending into the white matter either dorsally, dorsolaterally, laterally or ventrolaterally In all other directions dendrites disappeared to about the same degree

POSTNATAL DAY 14 (P14) Only a slight decrease in the amount of label was noted, resulting in a larger amount of label as on the EXT-side The difference was mainly due to dendrites extending dorsomedially into the medial part of lamina VI on the FLEX-side, which were absent on the EXT-side No change was observed in the dendritic extension pattern of the FLEX-MNs

POSTNATAL DAY 21 (P21, Fig 6) The amount of label continued to decrease slightly, mainly due to dendrites no longer extending ventromedially and medially No further changes were observed when compared to P14

POSTNATAL DAY 60 (P60) No changes were observed when compared to P21 Dendrites were found throughout C7 and C8 Dendrites extended mainly dorsomedially into the medial part of lamina VI, laterally into the lateral part of lamina VII and ventrally into the ventral part of lamina VII Relatively more label was found than on the EXT-side

Discussion

Methodological considerations

CTB-HRP provided reproducible results in this study, even though labelled MNs were found in spinal cord segments rostral to C7 and C8 These MNs, however, could be clearly identified as being labelled through diffusion to more proximal muscles and were therefore omitted in the present analysis Diffusion of the tracer in the distal forepaw muscles was confined to those parts of the muscles bordering on the muscle injected, i e either other digital and carpal flexors or extensors respectively as was shown by immunological detection of CTB-HRP in transverse sections of the paw (results not shown)

Both our results and the data presented by other authors (Beattie et al , 1990, Goldstein et al , 1990, Mong, 1990, Liang et al , 1991, Hirakawa et al, 1992, Ritz et al , 1992) show extensive labelling of the dendritic fields of MNs after injecting different variants of CT into several muscles As was shown by Ritz et al (1992) the labelling after injection of CT-HRP resembles the patterns after intracellular HRP-injections

Number of MNs

In the present study a slight non-significant decrease in postnatal MN numbers was noted (results not shown) which is in line with other studies (Janjua and Leong, 1984, Hardman and Brown, 1985, Bennet et al , 1986, Oppenheim, 1986)

Position of MNs

The position of MNs in the cervical spinal cord pro-

jecting to FLEX- or EXT-muscles has not previously been investigated in the rat as it has been in several other mammals (cat Fritz et al., 1981, 1982, 1986a, 1986b; monkey. Jenny and Inukai, 1983, dog· Mutai et al , 1986). The relative transverse positions of the two groups of MNs described in the present paper (EXT-MNs against the border between white and grey matter and FLEX-MNs in general medial to EXT-MNs) are in accordance with these data. The longitudinal position however differs. The above mentioned authors found MNs projecting to both FLEX- and EXT-muscles one segment further caudally than we did. This shift of approximately one segment in rat and also mouse versus cat, dog and monkey appears to be structural when corresponding muscles and nerves are compared (Baulac and Meininger, 1979, 1980; Pollin et al., 1990; versus Sterling and Kuypers, 1967; Thomas and Wilson, 1967; Fritz et al., 1981, 1982, 1986a, 1986b; Jenny and Inukai, 1983, Alstermark and Kummel, 1986; Mutai et al., 1986), a phenomenon also noticed in the lumbar spinal cords of rat and monkey by Janjua and Leong (1984).

Although no information is present to date about the postnatal positional changes of MNs in the cervical spinal cord of the rat, several studies concerning other species and/or other parts of the spinal cord report on the constant relative positions of MNs along both the longitudinal and the transverse axes (Baulac and Meininger, 1983, Smith and Hollyday, 1983; Janjua and Leong, 1984; Ulfhake et al., 1988, Ramirez and Ulfhake, 1991; Tanaka et al , 1992)

Development of the dendritic field

The adult pattern of the dendritic field of MNs develops predominantly postnatally as can be deduced from studies on longitudinally directed dendritic bundles (Scheibel and Scheibel, 1970, 1971; Bellinger and Anderson, 1987a, 1987b, Cameron et al., 1991; Lindsay et al., 1991; Westerga and Gramsbergen, 1992) It was further shown that in the cat the maturation of the dendritic bundles in the lumbar spinal cord lags approximately two weeks behind that of the cervical spinal cord (Scheibel and Scheibel, 1970, 1971). Such a difference in time-scale was also described by Ulfhake and Cullheim (1988) within the lumbar spinal cord of the cat: maturation of dendrites of intrinsic footsole MNs lags behind that of triceps surae MNs Therefore, it can be concluded that both a rostrocaudal and a proximodistal gradient exists, which is also present at all stages of prenatal MN differentiation (Altman and Bayer, 1984) In the present study a mediolateral gradient was also found: the postnatal maturation of EXT-MNs lags behind that of FLEX-MNs, which may be attributed to the fact that FLEX-muscles are innervated at an earlier stage than EXT-muscles (Angulo y Gonzalez, 1940, Altman and Bayer, 1984)

We have noted an increase in the amount of label followed by a decrease later in development. Since the number of MNs remains constant throughout postnatal development it is reasonable to assume that the number of dendrites initially increases and then decreases The increase is in agreement with the data from other workers who reported that this rise was always due to an increase in the number of second or higher order dendrites and never to the formation of new first order dendrites (Ulfhake et al , 1988; Cameron and Fang, 1989, Cameron et al., 1991; Ramirez and Ulfhake, 1991). We further noted an increase in dendritic extension followed by, in some directions, a decrease. In the literature, the data on dendritic extension during postnatal development vary considerably It was found to decrease (Ramirez and Ulfhake, 1991), to increase (Cameron et al., 1991), to initially increase and then to remain constant (Ulfhake et al , 1988) or to initially increase and then to decrease (Goldstein et al , 1990)

The dendritic extension patterns of different MN populations show a great diversity depending on muscle function and the synaptic input they receive. When a strong coordination between bilateral muscles is needed, contralaterally extending dendrites were found (bulbocavernosus: Goldstein et al., 1990; phrenicus: Lindsay et al., 1991; the tail. Ritz et al , 1992). In MNs mediating the toe-extension reflex, extensive dorsomedially extending dendrites in the direction of the primary afferents were found (Egger et al , 1980). Up until now, the dendritic extension pattern was never found to change during postnatal development (Goldstein et al., 1990; Cameron et al., 1991; Ramirez and Ulfhake, 1991) Thus, the present study is the first to describe a change in the directions in which dendrites extend in the transverse plane.

In conclusion, we have shown that the MN pools supplying two antagonistic muscles have a different postnatal developmental path to reach their specific adult dendritic extension pattern and, in particular, that the maturation of EXT-MNs lags behind that of FLEX-MNs. Both start at P2 with their dendrites extending in all directions, then follows a period of increase both in dendritic extension (which is more than the growth of the spinal cord itself) and in dendritic number, after which a period of specific dendritic retraction is found in which presumably, dendrites that did not form functionally significant contacts are eliminated. Among the latter are dendrites extending into the white matter of the lateral funiculus It was further shown that both MN pools exhibit a different extension pattern. Both popula-

tions have a ventral component directed towards MNs of axial and more proximal limb muscles, but EXT-MNs have a medially and FLEX-MNs a laterally directed component, which may point to a reciprocal connection between the two antagonist muscles. From P2 onwards, both have a dorsomedial component directed towards interneurons and primary afferents, however in FLEX-MNs this extension reaches as far as the medial part of lamina VI while EXT-MNs dendrites only extend temporarily as far as the lateral part of lamina VI. This distinction may point to a different way of innervation of the MNs, for example by the CST.

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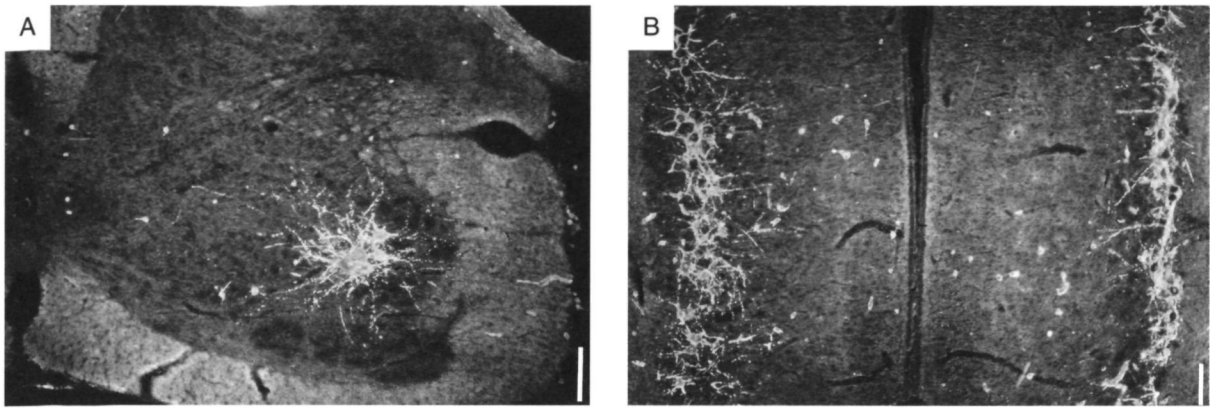

Fig. 1. Photomicrographs of sections of the cervical spinal cord of the rat under dark-field illumination showing the extensive labelling of the motoneurons and their dendritic fields obtained after intramuscular injections of CTB-HRP in the forepaw muscles of the rat. (A) Transverse section of the seventh cervical spinal cord segment at postnatal day 10 after injecting the FLEX-muscle. Lateral is to the right and dorsal to

the top. Scale bar = 100 μm. (B) Horizontal section through the seventh and eighth cervical spinal cord segments at the level of the central canal at postnatal day 7. Top is rostral. On the left side FLEX-MNs are labelled and on the right side EXT-MNs. Scale bar = 100 μm.

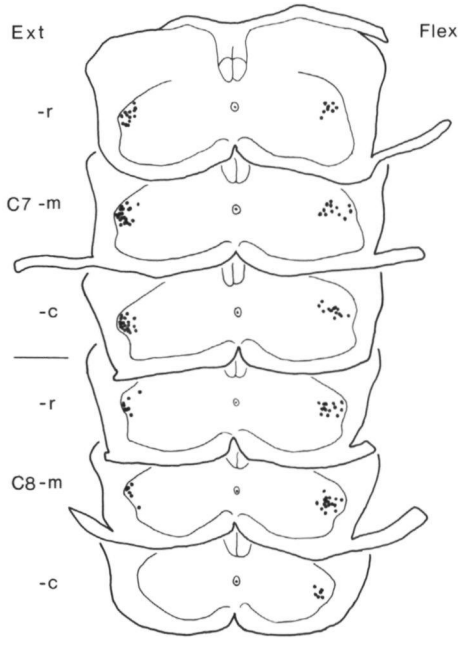

Fig. 2. Composite illustration of the seventh and eighth cervical spinal cord segments at postnatal day 2 showing the position of the EXT- (left side) and FLEX- (right side) MN somata after intramuscular injections of CTB-HRP. Both segments were divided in three equal parts: a rostral (-r), middle (-m) and caudal (-c) third. The data of all sections were pooled for their respective thirds.

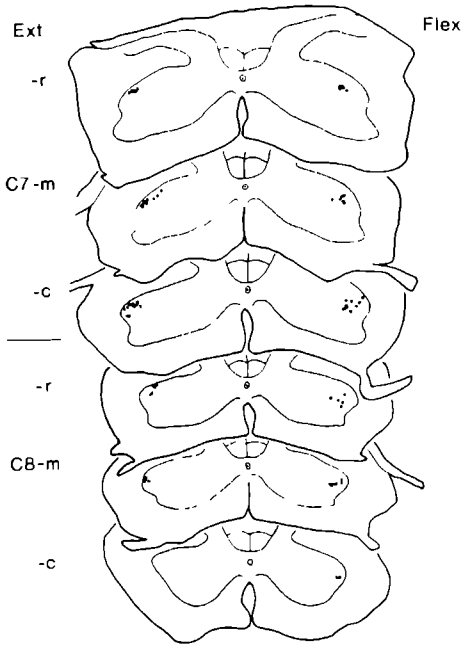

Fig. 3. Composite illustration of the seventh and eighth cervical spinal cord segments at postnatal day 21 showing the position of the EXT- and FLEX-MN somata. See Fig. 2 for full explanation.

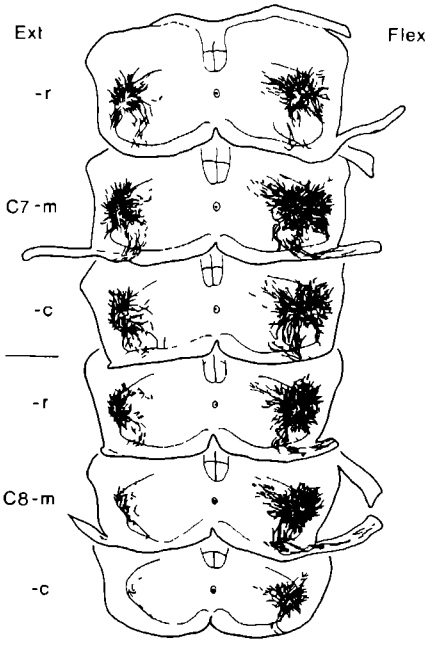

Fig. 4. Composite illustration of the seventh and eighth cervical spinal cord segments at postnatal day 2 showing the dendritic extension pattern of the EXT- (left side) and FLEX- (right side) MNs after intramuscular injections of CTB-HRP. Both segments were divided in three equal parts: a rostral (-r), middle (-m) and caudal (-c) third. The data of all sections were pooled for their respective thirds.

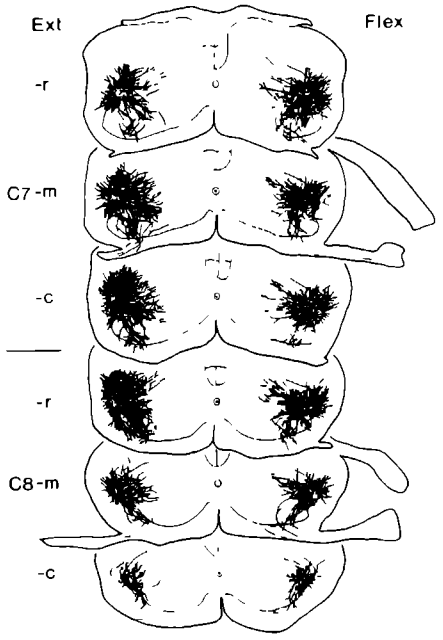

Fig. 5. Composite illustration of the seventh and eighth cervical spinal cord segments at postnatal day 7 showing the dendritic extension pattern of the EXT- (left side) and FLEX- (right side) MNs. See Fig. 4 for full explanation.

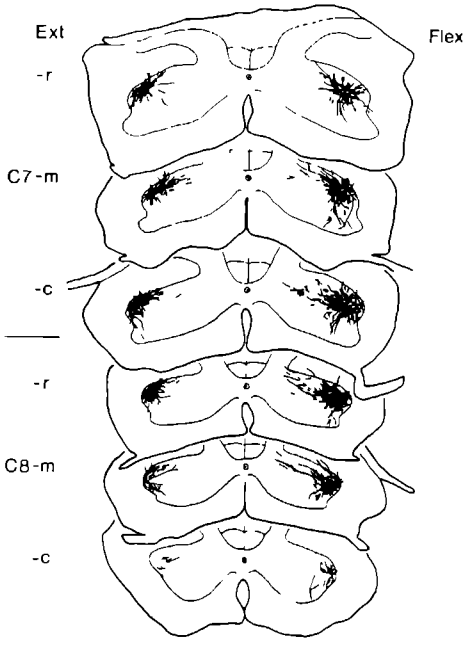

Fig. 6. Composite illustration of the seventh and eighth cervical spinal cord segments at postnatal day 21 showing the dendritic extension pattern of the EXT- (left side) and FLEX- (right side) MNs. See Fig. 4 for full explanation.

29 *Transient fields of termination of the developing rat corticospinal tract in the lower cervical spinal cord as demonstrated by anterograde tract-tracing using HRP*

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Summary

In the present paper a description is given of the development of the rat corticospinal tract (CST) in the lower cervical spinal cord This area contains, among other cells, the motoneurons innervating the distal forelimb muscles HRP gels were implanted in the sensorimotor cortex of Wistar rats varying in age from postnatal day 0 (P0) to P60 After a survival period of 48 hr the rats were transcardially perfused, the spinal cords transversely sectioned at 30 μm and the sections reacted for HRP Labelled CST axons in the dorsal funiculus were first detected at P2, and after a delay of 2 days the first fibres were found in the adjacent gray matter (P4) More labelled fibres were gradually added until maximal number and extension was reached at P10 By then the entire gray and large parts of the white matter were covered by labelled CST axons From P10 onwards the number of labelled CST fibres as well as their extension decreased In the adult rat some areas such as the lateral part of the ventral and dorsal horn and large parts of the ventral and lateral white matter ultimately became devoid of labelled CST axons It is concluded that a massive overshoot occurs during the devel-

opment of the terminal field of the rat CST. The results are discussed in conjunction with our previous findings on the development of the motoneurons innervating the rat distal forelimb muscles. The concurrent selective elimination of both CST axons and motoneuron dendrites is suggested to be correlated with progressively more mature, coordinated movements and with high digital skills especially.

Introduction

In the developing central nervous system outgrowing neurites are capable of finding their appropriate targets, even over long distances, and forming functional contacts whereas adult neurons no longer possess this capacity. A better understanding of the underlying events might therefore advance strategies for achieving functional recovery after damage to the mature nervous system. The rodent corticospinal tract (CST) provides an excellent model for the study of developmental events since its outgrowth into the spinal cord occurs postnatally (de Kort et al., 1985; Donatelle, 1977; Gribnau et al., 1986; Joosten and Gribnau, 1989; Joosten et al., 1987; O'Leary and Terashima, 1988; Schreyer and Jones, 1982) and is therefore accessible for experimental manipulations.

In the past the ontogenesis of the CST received much attention using various species and techniques as was recently reviewed by Stanfield (1992). Several processes taking place during the development of the CST and the cortical layer Vb neurons, from which the CST originates, have been described. Among them are neuron generation, cell migration, and cytodifferentiation, as well as cell death, and axon outgrowth into the brain and spinal cord white matter. However, the scarce data concerning the CST outgrowth into the spinal gray matter and the finding of the appropriate target are still controversial. In the young cat overgrowth of the target was observed: in some spinal gray areas CST axons were found which disappeared later on during development (Alisky et al., 1992; Theriault and Tatton, 1989). Although in the rodent some aberrant cortical areas transiently send axons into the spinal dorsal funiculus (Gorgels, 1990; Gorgels et al., 1989; Joosten et al., 1987; Joosten and van Eden, 1989; Stanfield and O'Leary, 1985; Stanfield et al., 1982; Uozumi et al., 1988; reviewed by Stanfield, 1992) no such overshoot of CST fibres was found in the spinal gray matter (Donatelle, 1977; Reh and Kalil, 1981). However, as was also noted by Stanfield (1992), this difference could be due to experimental variations instead of interspecies differences. On that account, we analyzed the projection areas of the CST in the rat spinal gray matter during development.

In addition, we wanted to gain insight in CST target finding and the influence of the target on that process. Lesion of the CST results in the impairment of the fine volitional digital flexion movements used for example for grasping small morsels of food (Castro, 1972a, b; Castro-Alamancos and Borrell, 1993; Montoya et al., 1991; Whishaw and Kolb, 1988). Therefore, in a previous study (Curfs et al., 1993), we examined the postnatal development of motoneurons (MNs) innervating the flexor digitorum muscle in the distal forelimb, a possible target for the CST. MNs innervating the antagonist extensor digitorum muscle were also studied. In order to correlate MN and CST development the present study was focused on the cervical spinal cord segments 7 and 8 (C7 and C8 respectively) since in these two segments the above mentioned MNs and their dendrites are located.

Materials and methods

Animals

Postnatal Wistar rats (Central Animal Laboratory, University of Nijmegen) of either sex, varying in age from the day of birth (postnatal day 0, P0) to young adult (P60) were used. Per age group at least 4 animals were examined; the ages of the rats in this paper are the ages at their respective days of sacrifice.

Labelling of the CST using HRP

In order to obtain maximal and stabilized CST labelling, the three steps incubation method, as introduced by Joosten (1991) for the labelling of CST growth cones, with some modifications was used. After anaesthesia with sodium pentobarbital (increasing with age 18-60 mg per kg body weight, i.p.), the skin over the skull was incised. Using a fine needle 3-6 (increasing with age) small holes were made into the skull and the underlying cerebral cortex encompassing the entire sensorimotor cortex. In order to label as many cortical neurons as possible, HRP (Boehringer Mannheim, grade 1)-gels (Griffin et al., 1979) were implanted, resulting in a gradual release of the tracer.

In a series of experiments the optimal postimplantation survival time was established to be 48 hours. Thereafter the rats were reanaesthetized (sodium pentobarbital, 25-90 mg per kg body weight, i.p.) and transcardially perfused with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 1% paraformaldehyde, 2% glutaraldehyde and 5% sucrose in 0.1 M phosphate buffer (PB, pH 7.4). After the perfusion the brain and spinal cord were dissected from the skull and spine respectively, and postfixed by immersion for 2 hours in the above mentioned fixative. The tissue was then cryoprotected by

immersion overnight in 20% sucrose in PB and embedded in 15% gelatin in the same solution Using a freezing microtome 30 µm sections were cut in either the horizontal or the transverse plane

Every horizontal and every fourth (P2-P21) or eighth (P60) transverse section was reacted for HRP-histochemistry The following 3-step procedure was used (1) tetramethylbenzidine-ammoniumheptamolybdate (TMB-AHM) incubation, next (2) diaminobenzidine-nickel (DAB-Ni) stabilization, followed by (3) DAB-cobalt-glucose oxidase intensification (DAB-Co-GO) Sections were rinsed in 0 1 M PB (pH 6 0), presoaked in TMB-AHM medium (5 0 mg TMB and 250 mg AHM in 100 ml 0 1 M PB, pH 6 0), and reacted for HRP by adding 50 µl 30% H_2O_2 every 5 min for a total of 20 min After rinsing in 0 1 M PB (pH 6 0) and 0 1 M PB (pH 7 4) the sections were incubated in DAB-Ni medium (20 mg DAB, 300 mg ammonium nickel sulphate and 10 µl 30% H_2O_2 in 100 ml 0 05 M Tris-HCl, pH 7 6) for 20 min The sections were rinsed in 0 05 M Tris-HCl and incubated in 0 5% $CoCl_2$ in 0 05 M Tris-HCl for 10 min, rinsed in 0 05 M Tris-HCl and 0 1 M PB (pH 7 4), and incubated in DAB-GO medium (50 mg DAB, 200 mg ß-D(+)-glucose, 40 mg NH_4Cl and 0 3 mg GO in 100 ml 0 1 M PB) for 1 h at 37µC After final rinses in 0 1 M PB the sections were mounted onto glass slides and air dried Following counterstaining with neutral red the sections were dehydrated and coverslipped with Depex

The spinal cord segments C7 and C8 were examined under dark field illumination Photomicrographs of representative sections were made using an automatic Zeiss photomicroscope II Using a Zeiss microscope equipped with a drawing tube, schematized drawings of the sections were made In these drawings the most important results were summarized The field of labelling in the spinal gray was subdivided into three parts a densely, an intermediately and a lightly labelled section respectively Also the position of the lateral motor column (LMC) was drawn

Results

Implantation of HRP-gels in the cortex results in a gradual diffusion of the HRP into the surrounding tissue yielding a protracted period of uptake of the tracer Forty-eight hours after implantation almost the entire cortical hemisphere was labelled including the fore- and hindlimb representations which project to the cervical and lumbar spinal cord respectively (Akintunde and Buxton, 1992, Castro-Alamancos and Borrell, 1993, Elger et al , 1977, Janzen et al , 1977, Joosten et al , 1987, Neafsey et al , 1986, Uozumi et al , 1988) Labelled cortical fibres could be followed throughout the brain and with progressive age into the spinal cord Extension of CST axons into the spinal cord gray matter was always maximal and reproducible after a survival period of 48 hours The results obtained from transverse sections were reassessed using horizontal sections

At the age of 2 days postnatally (P2) a heavily labelled main CST bundle was found in C7 and C8 in the contralateral dorsal funiculus The labelling was confined to the white matter, no labelling was found in the spinal gray (results not shown) In the ipsilateral ventral funiculus labelled CST axons were also encountered at every age studied This uncrossed CST component is well documented by other authors (Goodman et al , 1966, Joosten et al , 1992, Liang et al , 1991) and is left out of consideration in the present paper

At P4 the first labelled CST axons were found extending into the adjacent gray matter both in C7 and in C8 These axons were located within approximately 20 µm of the dorsal funiculus (Figs 1A, 2A, 4A)

At P7 labelled CST axons covered almost the entire contralateral gray matter (Fig 1B) except the most lateral parts of the superficial layers in the dorsal horn (Fig 2B) Besides, small sections of the ventral and lateral parts of the LMC in C7 and C8 respectively were devoid of label (Fig 3A) The densely labelled section was confined to the intermediate zone and reached laterally into the lateral funiculus This area was surrounded by the intermediately labelled section, which reached into the medial part of the dorsal horn and the dorsomedial part of the ventral horn Around the intermediately labelled area a lightly labelled section was encountered This area covered the lateral part of the dorsal horn, the ventrolateral part of the ventral horn, including almost the entire LMC, and extended into the lateral and ventral white matter as summarized in Fig 4B We also found labelled CST axons in the ipsilateral spinal gray projecting through the contralateral dorsal funiculus from P7 onwards This component however is left out of consideration in the present paper

At P10 labelled CST fibres covered the entire gray matter, including the complete LMC (Figs 1C, 2C, 3B) They also extended further into the ventral and lateral white matter than at P7 The densely labelled section was confined to the intermediate zone as at P7, it reached however less far into the lateral white matter (especially in C8) The outer boundaries of the intermediately labelled area were unchanged except for the less extending ventral one when compared to P7 The lightly labelled section

though extended further in all directions as summarized in Fig 5A

At P14 labelled CST fibres reached less far into both the gray and white matter when compared to P10 (Figs 1D, 2D, 3C) The densely labelled section was confined to the intermediate zone and no longer extended into the white matter The intermediately labelled area reached less far radially both into the white and into the gray matter than at P10 The lightly labelled section extended less far in all directions At this age large parts of the ventral and lateral white matter, and also the ventrolateral part of the LMC were devoid of labelled CST axons both in C7 and in C8 as summarized in Fig 5B

At P21 a further decrease of the extension of labelled CST axons was noted (Figs 1E, 2E, 3D) The densely labelled section was confined to the medial parts of the intermediate zone The intermediately labelled region reached less far laterally into the white and gray matter and also the lightly labelled section extended less far laterally and ventrally than at P14 The latter reduction was especially prominent in the lateral part of the LMC in C7, in the lateral parts of the dorsal horn and in the lateral white matter in C7 and C8 as summarized in Fig 6A

At P60 the extension of labelled CST fibres was further reduced (Figs 1F, 2F, 3E, 3F) The densely labelled section in C7 was confined to the medial and intermediate thirds and in C8 to the medial third of the intermediate zone The intermediately labelled region remained unchanged in C7 when compared to P21, in C8 it extended less far laterally, covering a smaller part of the white matter The lightly labelled section extended less far ventrolaterally both in C7 and C8 The LMC now only contained a few labelled fibres in the dorsomedial part as summarized in Fig 6B

Discussion

A prolonged supply of HRP to layer V pyramidal cells in the sensorimotor cortex through gels combined with a three-step incubation procedure results in the complete filling of CST axonal growth cones (Joosten, 1991) The same anterograde tracing method was used in the present investigation and it is therefore reasonable to assume that the entire CST terminal fields were labelled

At P2 labelled CST axons were present in the contralateral dorsal funiculus in C7 and C8, the youngest age that could be investigated with the technique used This agrees well with the data in literature on the developing rat CST (reviewed by Stanfield, 1992) The first CST fibres apparently arrive in the rostral cervical spinal cord at the day of birth (P0), in the caudal cervical spinal cord at P1 and in the rostral thoracic spinal cord at P2 (de Kort et al , 1985, Donatelle, 1977, Gribnau et al , 1986, Joosten and Gribnau, 1989, Joosten et al , 1987, O'Leary and Terashima, 1988, Schreyer and Jones, 1982) Shortly after these pioneer fibres have arrived more axons are gradually added to the CST, a process that continues until approximately P10 (Gorgels et al , 1989, Gribnau et al , 1986, Joosten and Gribnau, 1989, Schreyer and Jones, 1982)

In the present investigation, the first outgrowth of labelled CST fibres into the adjacent spinal gray was found at P4 This is in line with previous studies which reported of a delay of two days between the arrival of labelled CST axons at a given spinal cord segment and their first outgrowth into the adjacent spinal gray (Donatelle, 1977, Gribnau et al , 1986, Joosten et al , 1987, Schreyer and Jones, 1982) This 'waiting period' is caused by interstitial budding of the outgrowing CST axons (Gribnau et al , 1992) and was also demonstrated in the corticopontine projection (O'Leary et al , 1990, O'Leary and Terashima, 1988)

At P4 labelled CST axons extend in the direct vicinity of the main bundle Massive outgrowth into the spinal gray occurred from P4 onwards and maximal extension up into the white matter was reached at P10 Outgrowth of CST axons into the spinal gray occurred almost concentrically when the most distal boundary is concerned Thus CST axons seem to extend laterally with no directional preference, resulting in almost the entire contralateral spinal cord being labelled However, regional differences in the amount of label were noted Most CST axons were found in the intermediate zone and medial part of the lateral funiculus whereas fewer fibres were present in the medial part of the dorsal horn and the dorsomedial part of the ventral horn Even less fibres were encountered in the lateral part of the dorsal horn, the ventrolateral part of the ventral horn and the ventral and lateral white matter When compared to previous studies, we found labelled CST axons extending further, e g covering the entire LMC This difference can be ascribed to the higher accuracy of the HRP technique as compared to the proline technique used by Donatelle (1977) and the prolonged survival time of 48 hours as opposed to the survival time of 24 hours used by Joosten et al (1987) It is very unlikely that the larger CST terminal fields found in the present investigation must be attributed to transneuronal transport of the HRP Firstly, no such anterograde transport has been reported for HRP and secondly no labelled somata were found, not even with longer survival times (72 hours, personal observation)

The most striking finding of the present study is

the overgrowth of CST fibres both numerically and spatially up to P10 inclusive, followed by a selective elimination during further development In the second and third postnatal weeks CST fibres are gradually withdrawn from the ventral and lateral white matter as well as the lateral dorsal horn and the ventrolateral and lateral parts of the LMC The latter structure only retains a few CST axons in its dorsomedial part in the adult stage The numerical decline can easily be deduced from the progressively diminishing densely labelled section as illustrated in Fig 1 The adult CST terminal field appeared to be larger than that reported in the literature which may be attributed to the higher sensitivity of the HRP method (Antal, 1984, Donatelle, 1977, Gribnau and Dederen, 1989, Liang et al , 1991), the intensifying incubation method (Schreyer and Jones, 1982), and the prolonged survival time (Gribnau et al , 1986, Joosten et al , 1987) Exuberant outgrowth of neurites followed by a period in which redundant processes are eliminated except those making functional contacts is a common feature in nervous system development This phenomenon has been described previously not only for CST outgrowth in the rat dorsolateral funiculus (Gorgels, 1990, Gorgels et al , 1989, Joosten et al , 1987, Joosten and van Eden, 1989, Stanfield and O'Leary, 1985, Stanfield et al , 1982, Uozumi et al , 1988, reviewed by Stanfield, 1992), and for the developing CST in other mammals (Alisky et al , 1992, reviewed by Kalil, 1988, Stanfield, 1992) but also for other developing long axonal pathways in rats (McConnell et al , 1989, O'Leary and Terashima, 1988) Selective eliminatory events were also noted during MN dendritic field maturation (Curfs et al , 1993, Goldstein et al , 1990, Ramirez and Ulfhake, 1991) and during MN development in general (Brown and Booth, 1983, Fraher and O'Sullivan, 1989, Lance-Jones, 1982, reviewed by Oppenheim, 1989) The present study, however, is the first to report on the existence of transient CST axons in the rat cervical spinal cord gray matter during postnatal development Obviously the less economic strategy of overshoot during development is preferred above straight forward outgrowth according to a 'blue-print' The reason can to date only be speculated at, however this mechanism provides the developing brain and spinal cord with plasticity to react to environmental fluctuations eventually resulting in an optimally adapted central nervous system It should be noted that the elimination of CST neurites and of MN dendrites occurs from the second postnatal week onwards (Caroni and Becker, 1992, Curfs et al , 1993), i e the period during which more mature locomotor behaviour develops (Eilam and Golani, 1988, Prendergast and Shusterman, 1982, Westerga and Gramsbergen, 1990, 1993) From these concurrent events the assumption can be made that the development of the CST terminal field at least in part depends upon the formation of functional contacts The resemblance of rat CST development to that in other species such as the cat and opossum (Alisky et al , 1992, Cabana and Martin, 1985, Theriault and Tatton, 1989) points to common general ontogenetic events in mammals

The adult rat fits into the group of mammals (such as raccoon, slow loris, galago and rhesus monkey, reviewed by Armand, 1982) characterized by high digital skills and direct CST innervation of the cervical LMC (Elger et al , 1977, Fritz et al , 1985, Liang et al , 1991, reviewed by Porter, 1987) as opposed to those mammals with no direct projection to the LMC and low digital skills, such as the cat (Alisky et al , 1992, Alstermark and Sasaki, 1985, Fujito et al , 1991, Illert and Wiedemann, 1984, Satomi et al , 1991, reviewed by Armand, 1982, Heffner and Masterton, 1975, Kuypers, 1982) From the present study it can be deduced that the developing CST is able to innervate, possibly transiently, many neuron populations in the rat lower cervical spinal cord The adult CST innervates interneurons (both sensory and premotor interneurons), and probably also MNs (Elger et al , 1977, Liang et al , 1991) In a previous paper we have shown that the developing dendritic fields of MNs innervating the extensor and especially the flexor muscles of the rat distal forelimb cover large parts of the ventral horn in C7 and C8 (Curfs et al , 1993) Obviously the CST terminal field and the MN dendritic fields in C7 and C8 have large areas of overlap Current research is focused on the development of synaptic contacts between the CST and MNs through double labelling at the ultrastructural level which may reveal transient synaptic contacts during development

In the adult stage rat flexor MNs have a dorsomedially directed dendritic component which is lacking in the extensor MNs (Curfs et al , 1993) Besides, flexor MNs are located in the dorsomedial part of the LMC, i e the area that in the adult rat contains labelled CST axons whereas extensor MNs are located in the dorsolateral part of the LMC, i e the area ultimately devoid of labelled CST axons It can thus be speculated that MNs innervating flexor muscles in the distal forelimb receive more direct innervation from the CST than MNs of extensor muscles Further support for this hypothesis is obtained through lesion studies of the CST resulting in the impairment of especially the fine volitional digital flexion movements, used for example for grasping small morsels of food (Castro, 1972a, b, Castro-Alamancos and Borrell, 1993, Montoya et al , 1991, Whishaw and Kolb,

1988). Possibly, the property of high digital skills is correlated with species-specific ontogenetic features superimposed upon the general ontogenetic events mentioned above. Future comparative ontogenetic research in the cat may add proof to this hypothesis

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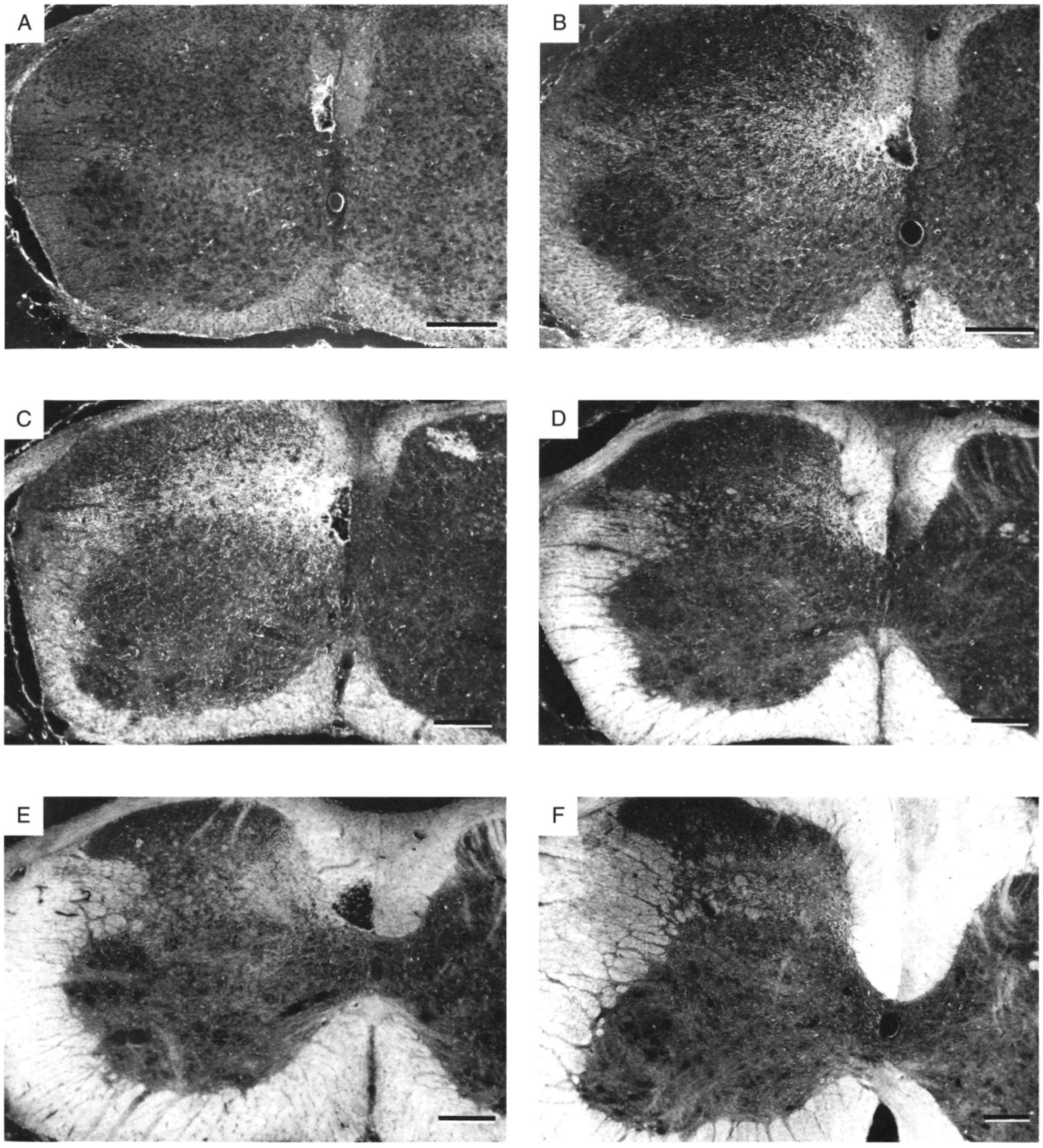

Fig. 1. Photomicrographs of transverse sections of the rat contralateral cervical spinal cord under dark-field illumination 48 h. after HRP-gel implantation in the sensorimotor cortex. Scale bar is 200 μm. A. C8 at postnatal day 4. B. C8 at postnatal day 7. C. C7 at postnatal day 10. D. C8 at postnatal day 14. E. C8 at postnatal day 21. F. C7 at postnatal day 60.

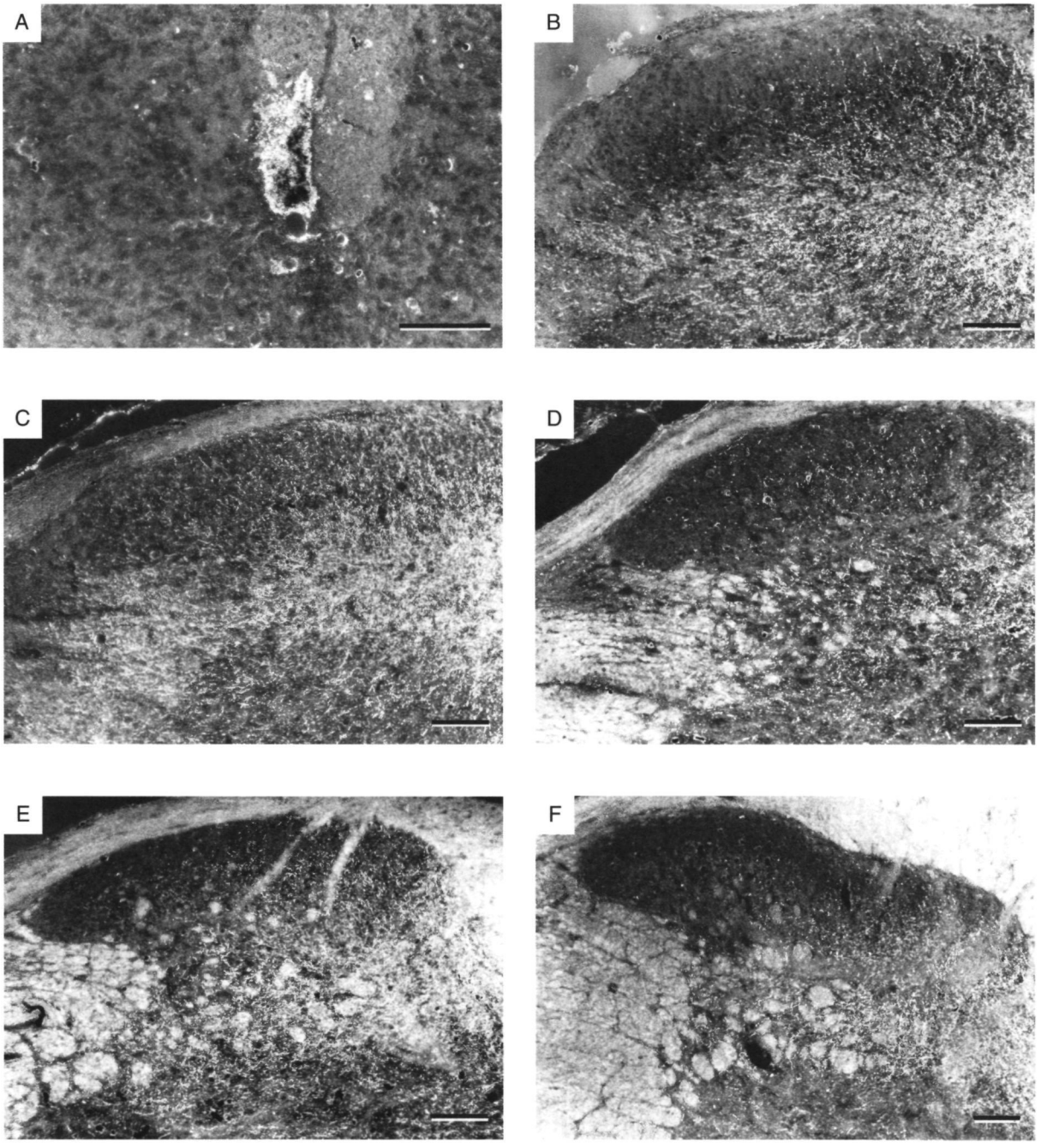

Fig. 2. Photomicrographs of transverse sections of the rat contralateral cervical dorsal horn under dark-field illumination 48 h. after HRP-gel implantation in the sensorimotor cortex. Scale bar is 100 μm. A. C8 at postnatal day 4. B. C8 at postnatal day 7. C. C7 at postnatal day 10. D. C8 at postnatal day 14. E. C8 at postnatal day 21. F. C7 at postnatal day 60.

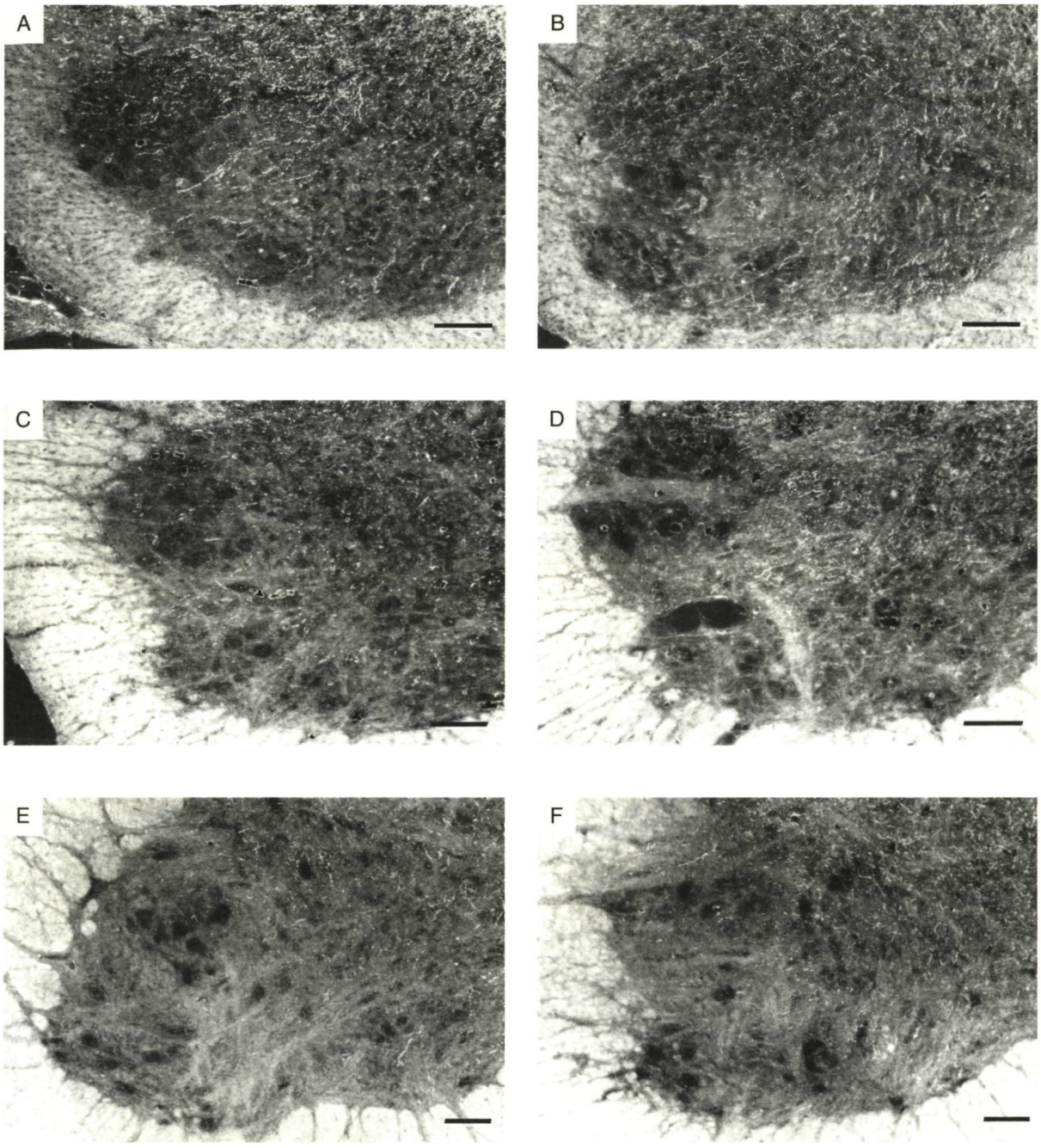

Fig. 3. Photomicrographs of transverse sections of the rat contralateral cervical ventral horn under dark-field illumination 48 h. after HRP-gel implantation in the sensorimotor cortex. Scale bar is 100 μm. A. C8 at postnatal day 7. B. C7 at postnatal day 10. C. C8 at postnatal day 14. D. C8 at postnatal day 21. E. C7 at postnatal day 60. F. C8 at postnatal day 60.

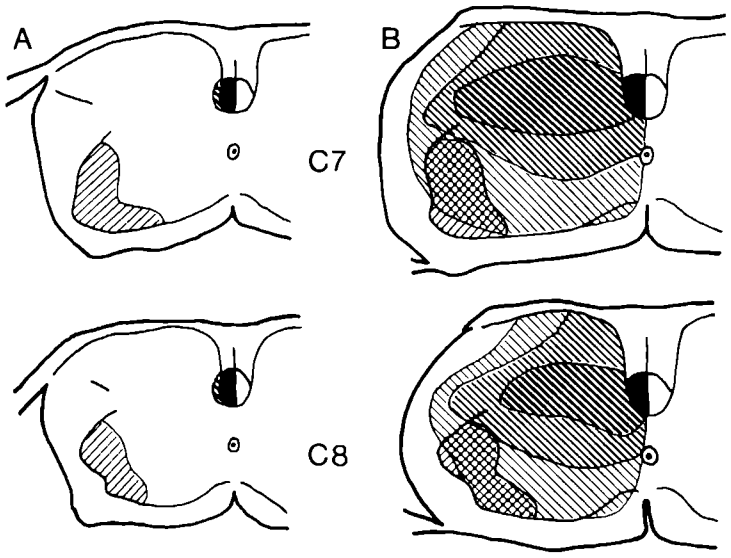

Fig 4. Schematized maps of transverse sections through the middle third of the cervical spinal cord segments 7 and 8 at P4 (A) and P7 (B) 48 h after HRP-gel implantation in the sensorimotor cortex Labelled CST fibres in the dorsal funiculus are indicated by the black filled area and in the spinal gray by the hatched area The densely, intermediately and lightly labelled region is indicated by dark, middle and light hatching, respectively. The position of the lateral motor column in the ventral horn is indicated by the hatching perpendicular to that of the CST

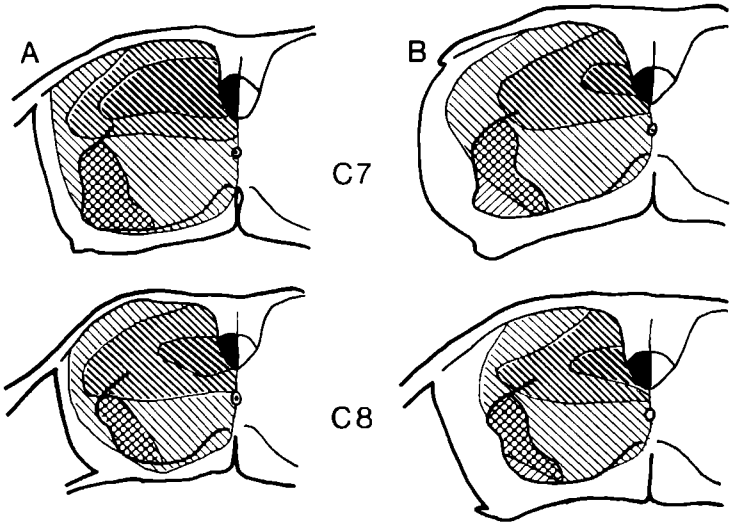

Fig 5. Schematized maps of transverse sections through the middle third of the cervical spinal cord segments 7 and 8 at P10 (A) and P14 (B) 48 h. after HRP-gel implantation in the sensorimotor cortex See Fig 4 for full explanation.

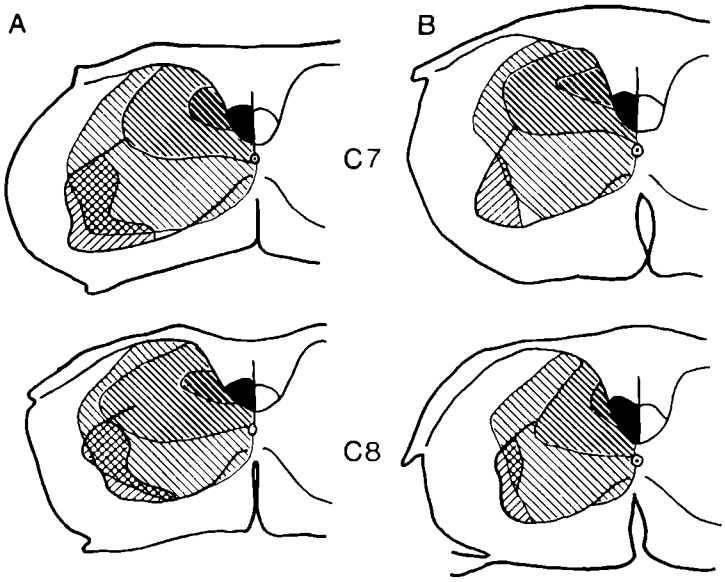

Fig. 6. Schematized maps of transverse sections through the middle third of the cervical spinal cord segments 7 and 8 at P21 (A) and P60 (B) 48 h. after HRP-gel implantation in the sensorimotor cortex. See Fig. 4 for full explanation.

41 *Direct cortico-motoneuronal synaptic contacts are present in the adult rat cervical spinal cord and are first established at postnatal day 7*

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Based on Neuroscience Letters, submitted

Key words Corticospinal tract, Motoneurons, Synaptic contacts, Cervical spinal cord, Double labelling, Electron microscopy, Rat

Summary

In order to demonstrate direct cortico-motoneuronal synaptic contacts in the cervical spinal cord of the rat and to determine at which postnatal age these contacts are established, an electron microscopic study using double labelling was made Corticospinal axons were anterogradely labelled after HRP-gel implantation into the cerebral motor cortex and motoneurons were retrogradely labelled after CTB-HRP injections into the distal forelimb flexor muscle With the histochemical procedures used, both tracers yield similar needle-like crystalline deposits Labelled axons however, can well be differentiated from labelled motoneuronal dendrites and somata on morphological grounds In adult rats, direct cortico-motoneuronal contacts were encountered Experiments in developing postnatal rats demonstrated that these synapses are first present on postnatal day 7

Introduction

The corticospinal tract (CST) is the latest central fibre system to develop in mammals In the rat, its outgrowth into the spinal cord, its subsequent target

finding and synaptogenesis even comes about postnatally (e.g. Gribnau et al., 1986; Schreyer and Jones, 1982; Stanfield, 1992). This makes it a suitable model in studying developmental events. Understanding of the events occurring during development of the central nervous system might provide strategies to increase the regenerative capacities of the brain and spinal cord after damaging. Previously, we have investigated the outgrowth of the CST into the cervical spinal gray and the CST fibres appeared to overgrow their target during the first postnatal week, whereas later on redundant fibres are eliminated (Curfs et al., 1994). Because of the involvement of the CST in especially the voluntary flexion movements of the distal forelimb (e.g. Castro, 1972a, b; Montoya et al., 1991; Schrimsher and Reier, 1993), we also examined the postnatal development of motoneurons (MNs) innervating the muscles involved (Curfs et al., 1993). It was shown that distal flexor MN dendrites increase in number and extension in the first postnatal week, followed by a decrease later on. From the two studies it was further concluded that the CST projection area largely overlaps these dendrites.

In order to investigate the influence of the target upon the development of the CST, it needs to be determined whether flexor MNs are a direct or an indirect target of the CST and thus the question arises whether direct synaptic contacts exist between the CST and flexor MNs. Although indications of the existence of direct synaptic cortico-motoneuronal contacts were presented electrophysiologically (Elger et al., 1977) and light microscopically (Liang et al., 1991), evidence at the electron microscopical level is still missing. In the present study, a method is presented to demonstrate these contacts in the adult rat, and in addition we investigated at what age these contacts are formed. Ideally, such a technique needs to fulfil four criteria. Firstly, CST axons and flexor MN somata and dendrites need to be labelled massively, making it easier to find synapses in ultrathin sections. Secondly, survival times need to be short, especially when a developmental study is performed. Thirdly, the ultrastructure needs to be sufficiently conserved to recognize synaptic structures. Fourthly, the labelling should allow discriminating between labelled axons and labelled somata and dendrites, respectively. Pilot studies revealed that only the combination of anterograde horseradish peroxidase (HRP) labelling in conjunction with retrograde cholera toxin subunit B conjugated to HRP (CTB-HRP) labelling, with the histochemical detection of HRP, fulfilled the first three criteria. Several other tracers proved to be less suitable, such as *Phaseolus vulgaris* leucoagglutinin (long survival times, bad ultrastructure), neurobiotin, wheat germ agglutinin, and dextran amines (all of them resulting in a small number of labelled fibres). A disadvantage of the method employed is that CST axons can not be discriminated from MN somata and dendrites on the basis of the kind of labelling, which in both cases consists of needle-like crystalline deposits. We demonstrate in this study, however, that this differentiation can easily be made on the basis of the ultrastructure of axons versus somata and dendrites, respectively.

Materials and methods

Postnatal Wistar rats (Central Animal Laboratory, University of Nijmegen) of either sex, varying in age from the day of birth (postnatal day 0, P0) to young adult (P60) were used. Per age group at least 3 animals were examined; the ages of the rats in this paper are the ages at their respective days of sacrifice.

Corticospinal axons were labelled by HRP-gel implantation, as previously described (Curfs et al., 1994). In brief, rats were anaesthetized with sodium pentobarbital (increasing with age: 18-60 mg per kg body weight, i.p.), the skin over the skull was incised and using a fine needle 3-6 (increasing with age) small holes were made into the skull and the underlying cerebral cortex encompassing the entire left sensorimotor cortex. HRP (Boehringer Mannheim, grade 1)-gels (Griffin et al., 1979) were implanted effectuating a gradual release of the tracer. Flexor MNs were labelled by CTB-HRP injection, as previously described (Curfs et al., 1993). In brief, the skin of the forepaw was incised ventrally to expose the right FLEX-muscles. Using a 5 µl Hamilton syringe fitted with a glass micropipette, 0.5 µl of a 0.1% CTB-HRP solution (List Biological) was pressure injected into the FLEX-muscles.

Since recurrent collaterals and sensory afferents from the injected muscle synapsing directly on the labelled MNs would lead to false-positive direct cortico-motoneuronal synaptic contacts, in each age-group animals in which the HRP-gel implantation into the cortex was omitted were also analyzed. Further processing was identical to the double-labelled material.

After the optimal survival time (48 h. for P0-P21, and 72 h. for P60 as previously determined) the rats were reanaesthetized (sodium pentobarbital, increasing with age: 25-90 mg per kg body weight, i.p.) and transcardially perfused with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 1% paraformaldehyde, 2% glutaraldehyde and 5% sucrose in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion the brain and spinal cord were dis-

sected from the skull and spine respectively, postfixed by immersion for 2 hours in the above mentioned fixative and immersed in 5% sucrose in PB (pH 7 4) Using a vibratome 50 µm sections were cut from cervical spinal segments 7 and 8 (C7 and C8, respectively) The sections were collected in PB (pH 7 4) and immediately processed according to the protocol of Joosten et al (1987) In brief, the sections were rinsed in PB (pH 6 0), pre-incubated in tetramethylbenzidine-ammoniumheptamolybdate medium (250 mg AHM and 5 mg TMB (dissolved in absolute ethanol) in 100 ml PB, pH 6 0) for 20 min The incubation was started by adding 50 µl 30% H_2O_2 per 100 ml pre-incubation medium every 5 min for a total of 20 min The reaction was terminated by rinses in PB (pH 6 0) Thereafter the sections were osmificated in 1% OsO_4 in PB (pH 5 0) for 4 h , rinsed in PB (pH 5 0), dehydrated in increasing concentrations of ethanol, rinsed in acetone, and finally embedded in Epon 812 on repelcoated slides After light-microscopic examination, selected sections were photomicrographed under bright field illumination using an automatic Zeiss photomicroscope II and further processed for electron microscopy

Semi-thin and ultra-thin sections were cut using a Reichert-Jung Ultracut microtome Semi-thin sections were counterstained with toluidine-blue and examined with a Zeiss light microscope to select the field of interest Ultrathin sections were collected on 75-mesh formvar coated copper grids, contrasted with uranyl acetate for 20 min and lead citrate for 10 min , and examined in a Philips EM 301 or a Jeol EM 1010 at an accelerating voltage of 60 kV

Results

Light microscopic analysis of the vibratome sections revealed that both the CST and the flexor MNs and their dendrites, respectively, were well labelled in the cervical spinal cord segments 7 and 8 (C7-C8) at all postnatal ages investigated including the adult The area containing the MN somata and presumed dendrites was trimmed and further processed for electron microscopy (Fig 1A)

Examination of ultrathin sections of single labelled material, i e from animals which received only CTB-HRP injections into the flexor muscle, never revealed double labelled synapses at all ages investigated Up until P5, labelled axon terminals were found, probably belonging to primary afferents, but never in the area where labelled dendrites were encountered At later ages, the anterograde transport rate was likely to be too slow to label these axons in the field of interest It can thus be concluded that the double labelled synapses found after both anterograde and retrograde labelling reflect direct cortico-motoneuronal contacts

In ultrathin sections axons could be well discriminated from dendrites and somata on the basis of their ultrastructural morphology MN somata are characterized by, among others, clusters of ribosomes (Fig 1B), axon terminals by synaptic vesicles and mitochondria (Fig 1C, D), and dendrites by microtubular structures and a relatively low amount of electrondense material (Fig 1C, D, Peters et al , 1991) Besides, the HRP crystals in general tended to be smaller in CST axons than in MN somata and dendrites This latter difference was, however, never used as an absolute criterium but only as additional evidence In adult rats, double labelled synapses could easily be detected They were located on flexor MN dendrites and were characterized by round vesicles (Fig 1D), and thus are most likely excitatory (Peters et al , 1991) Based on the overlap between labelled CST fibres and MN dendrites, which originates between P4 and P7 (Curfs et al , 1993, 1994), it was expected that cortico-motoneuronal contacts were generated during this time-range Examination of ultrathin sections at P5 revealed no double labelled synapses, although labelled CST axons were already present in the area of the labelled MN dendrites Double labelled synapses could first be found at P7 These were again located on flexor MN dendrites and contained round vesicles (Fig 1C)

Discussion

With the technique presented here, we were able to demonstrate the existence of monosynaptic contacts between the CST and MNs innervating the flexor muscles in the distal forelimb in the rat These contacts were located on MN dendrites as was already suspected from light microscopical evidence (Liang et al , 1991) It was further shown that these synapses are most likely excitatory, as based on the presence of round vesicles in the axon terminal (Peters et al , 1991) This is in agreement with the electrophysiological finding that stimulation of the rat motor cortex yields monosynaptic EPSPs and action potentials in MNs in the cervical spinal cord (Elger et al , 1977)

In addition, we have demonstrated that cortico-motoneuronal contacts are formed between P5 and P7, being the ages at which CST axons start to overlap with flexor MN dendrites (Curfs et al , 1993, 1994) It is reasonable to assume that at later ages more synapses are added since the terminal field of the CST still increases thereafter (Curfs et al , 1994) It would be very interesting to know if any transient contacts are formed between the CST and flexor MNs, in analogy to other fibre systems with their

respective targets (e.g. Goodman and Shatz, 1993; Kalb and Hockfield, 1992; Lichtman and Balice-Gordon, 1990; Lowrie and Vrbová, 1992; Navarette and Vrbová, 1993; Oppenheim, 1989). Using the expression of the immediate early gene c-fos after kainate stimulation of the motor cortex, transient contacts were found between the CST and spinal interneurons (Curfs et al., in press). The technique described in the present paper is suited for a detailed quantitative electron microscopical study in order to tackle this question.

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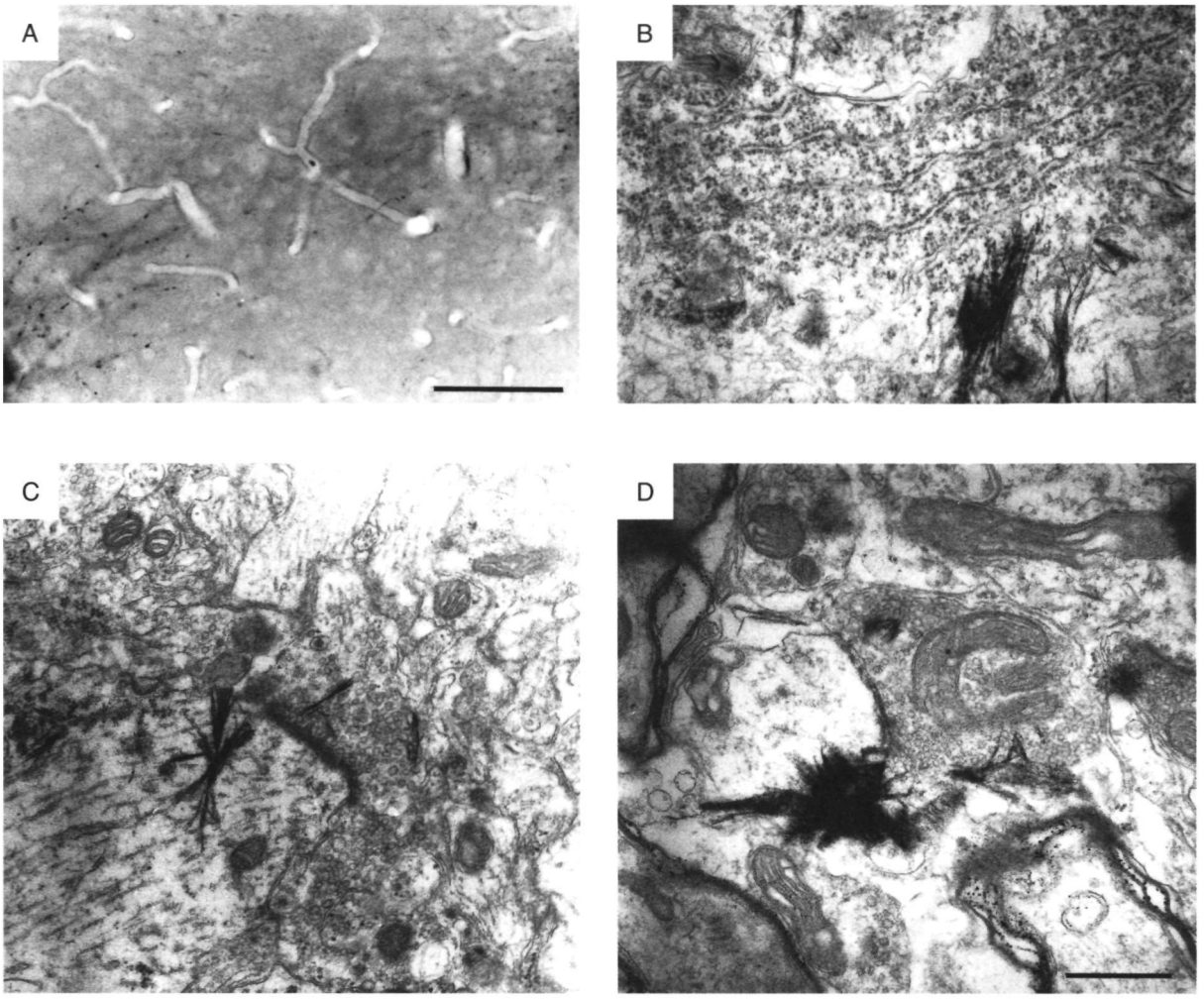

Fig. 1. A. Light microscopic photograph of a 50 μm vibratome section illustrating the anterograde HRP labelling of the corticospinal tract and the retrograde labelling of motoneurons and their dendrites innervating distal forelimb flexor muscles in cervical spinal cord segment 7 of rats at postnatal day 7. The labelled corticospinal tract in the dorsal funiculus is located in the top right corner and the labelled motoneuron somata in the bottom left corner. Scale bar is 100 μm. B. Electron microscopic photograph of a retrogradely labelled flexor motoneuron soma in cervical spinal cord segment 7 of an adult rat. C-D. Electron microscopic photograph of a labelled corticospinal axon terminal synapsing upon a labelled flexor motoneuron dendrite in cervical spinal cord segment 7 at postnatal day 7 (C) and of an adult rat (D). Scale bar in D applies to B-D = 0.5 μm.

47 *Transneuronal labelling of spinal interneurons after pseudorabies virus injections in distal forelimb muscles of maturing rats*

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Summary

We have previously shown, using retrograde labelling with cholera-toxin subunit B injections into the distal forelimb flexors of maturing rats, that the motoneurons innervating this muscle show specific changes in their dendritic field In the first postnatal week, a large increase is found in the number and extension of dendrites whereas in the following postnatal weeks dendrites are eliminated (Curfs et al , 1993) In the present study, we address the question whether this change in motoneuron dendrites is also reflected in the interneuron population innervating these motoneurons This population was investigated by means of transneuronal labelling after pseudorabies virus injections into the distal forelimb flexor muscles of rats at postnatal day 1, 7, and 21 Postinjection survival times were 24 h for the former and 48 h for the latter two After immunohistochemical detection of the pseudorabies virus most rats showed labelling of motoneurons in cervical spinal cord segments 7 and 8, located dorsolaterally in the lateral motor column In the majority of rats with motoneuronal labelling, also transneuronal labelling of interneurons in the cervical spinal cord was detected, mainly in the intermediate zone and the dorsal and medial part of the ventral horn As based on their location these interneurons probably reflect propriospinal interneurons in cervical spinal cord segments 2 to 4, whereas

in cervical spinal cord segments 5 to 8 several subpopulations mediating signals from various afferent systems were found. In spite of the large variation in labelling intensity, reflected mainly in the number of interneurons labelled, comparable interneuron subpopulations were labelled at all three postnatal ages analyzed. In addition, most animals also showed labelling of sympathetic preganglionic neurons in the intermediolateral cell column in thoracic spinal cord segments 2-5. No transneuronal labelling from this autonomic nucleus was found. It is thus concluded that pseudorabies virus injections into forelimb muscles can be used to map the interneuron populations in maturing rats and secondly, that the pattern of motoneuron innervation by interneurons, as based on the location of the latter, is already well established at birth.

KEY WORDS: Postnatal maturation; Cervical spinal cord; Interneurons; Pseudorabies virus; Rat

Introduction

In our program on the developing rat corticospinal tract (CST) we try to elucidate the mechanisms involved in the path and target finding of outgrowing corticospinal axons and the influence of the target upon these processes. Previously, we have described the maturation of the dendritic fields of MNs (Curfs et al., 1993), as a target of the CST (Elger et al., 1977; Liang et al., 1991) in correlation with the outgrowth of CST fibres into the spinal gray matter (Curfs et al., 1994). The dendritic fields of MNs expand during the first postnatal week, and later on during development redundant dendrites are eliminated. CST fibres spread over the spinal gray during the first one and a half week and thereafter transient fibres are eliminated. It was thus concluded that both events are correlated, at least when their timing is concerned.

The greater part of the innervation of the MNs by the CST however, will occur indirectly via spinal interneurons (INs). In order to study their influence, the INs in the cortico-motoneuronal pathway must first be mapped. In general, INs innervating MNs can be labelled after injection of wheat germ agglutinin, possibly conjugated to horseradish peroxidase (WGA-HRP), or tetanus toxin fragment C into the muscle in question. After being internalized in the neuromuscular junction, the tracer is retrogradely transported to the MN cell body and its dendrites, and eventually transneuronally to INs terminating upon these MNs (Alstermark et al., 1990, 1991; Alstermark and Kummel, 1986, 1990a, b; Collins et al., 1991; Manning et al., 1990; Moschovakis et al., 1992, among others). This technique has, however, two major disadvantages. Firstly, the labelling signal fades as higher order neurons become labelled, and secondly, in order to obtain transneuronal labelling, relatively long survival times are needed, whereas in developmental studies short survival times are crucial. Recently, transneuronally transporting viruses were introduced as neuroanatomical tracers (reviewed by Kuypers and Ugolini, 1990). Especially the herpes related pseudorabies virus (PRV) has since then frequently been used to retrogradely label chains of neurons involved in the innervation of various autonomic systems (Dobbins and Feldman, 1994; Haxhiu et al., 1993; Jansen et al., 1992; Loewy et al., 1994; Loewy and Haxhiu, 1993; Marson et al., 1993; Standish et al., 1994; Ter Horst et al., 1993; among others), but also muscle control (Rotto-Percelay et al., 1992). The major advantage of viral tract-tracing is that the virus replicates in the neuron, thereby amplifying its own signal also in transneuronally infected cells. In the present study we demonstrate that the spinal INs innervating MNs controlling distal forelimb muscles can be mapped in maturing postnatal rats.

Materials and methods

A total number of 20 Wistar rats (Central Animal Laboratory, University of Nijmegen) of either sex and of three different postnatal ages were used. The day of birth was designated as postnatal day 0 (P0), and the ages given here are the ages at their respective days of perfusion.

After anaesthesia with sodium pentobarbital (increasing with age 18-60 mg per kg body weight, i.p.) a small hole was made in the skin overlying the flexor muscles of the distal left forelimb using a fine needle, in order to prevent the spreading of the virus into the skin. Thereafter a glass pipette connected to a Hamilton syringe was inserted via the hole into the muscle, and 0.5 µl of a solution containing $3\mu10^6$ PFU/ml of Bartha's strain, an attenuated strain characterized by decreased virulence when compared to the wild-type form (Strack et al., 1989) of PRV (kindly donated by Dr. T.C. Mettenleiter, Tubingen, Germany) was pressure injected into the muscle belly. After the injection the needle was left in place for 1 min. and after retraction the wound was swapped clean.

Maximal survival times were assessed to be 24 h. and 48 h. for animals aged 1 and 7 days postnatally (P1 and P7), respectively. For animals aged 21 days postnatally (P21), the survival times were kept constant at 48 h. in order to achieve consistency. At these survival times, the majority of the rats showed no pathological symptoms of viral infection yet. Longer

survival times most likely resulted in large discomfort for the animals and eventually within another 24 h in their deaths The rats were reanaesthetized (increasing with age 25-90 mg sodium pentobarbital per kg body weight, i p) and transcardially perfused with ice-cold 0 1 M phosphate buffered saline (PBS, pH 7 4), followed by 4% paraformaldehyde in 0 1 M PB or 2% paraformaldehyde and 0 12% glutaraldehyde in PB After the perfusion the brain and spinal cord were dissected from the skull and spine, respectively, and postfixed by immersion in fixative for a least 3 h The tissue was then cryoprotected by immersion in 20% sucrose in 0 1 M PB The cervical and thoracic spinal cord was subdivided in blocks of 2 and 3 segments, respectively, and the contralateral side (i e the right side) was marked by perforation of the lateral funiculus with a needle Using a freezing microtome transverse 30 µm sections were cut and collected in a 1 out of 2 series in 0 1 M PBS Transverse sections of the brain were collected in a 1 out of 6 series

The presence of PRV in infected neurons was detected by immunohistochemistry Sections were rinsed twice in 0 1 M potassium phosphate buffered saline (KPBS, pH 7 4), incubated in 0 3% H_2O_2 for 10 min to block endogenous peroxidases, rinsed for three times in KPBS and incubated in 1% sodium borohydride for 6 min to block free aldehydes After five rinses in KPBS the material was pre-incubated for 1 h in KPBS containing 0 1% Triton, 0 1% bovine serum albumin and 2% normal goat serum (KPBS-BT-NGS) Then the sections were incubated overnight at room temperature with rabbit antibodies against PRV (kindly donated by Dr J Pol, CDI Lelystad, The Netherlands, dilution 1 2500, in KPBS-BT-NGS) After three rinses, the sections were incubated in a solution containing goat anti rabbit IgG's (1 200, Nordic Immunology, Tilburg, in KPBS-BT) for 1µ h, rinsed three times in KPBS, and incubated in rabbit peroxidase anti peroxidase complex (1 600, Dakopatts, in KPBS-BT) for 1µ h After two rinses in KPBS and two rinses in 0 05 M Tris buffered saline (TBS, pH 7 6) the peroxidase was revealed by a nickel intensified diaminobenzidine incubation (300 mg nickel ammonium sulphate, 20 mg diaminobenzidine, and 10 µl H_2O_2 in 100 ml TBS) for 3 min After two final rinses in TBS, the sections were mounted using a gelatin chrome alum solution, air dried, dehydrated and coverslipped with Depex

Drawings of the sections were made using a Zeiss microscope equipped with a drawing tube Labelled neurons in one spinal segment were pooled into a representative drawing and a composite reconstruction of the spinal cord was made Photomicrographs were made using an automatic Zeiss photomicroscope II

Results

Labelling of motoneurons in the cervical spinal cord
Almost all animals showed labelling of the presumed MNs in cervical spinal cord segments 7 and 8 (C7-8), as based on their location when compared to injections of cholera toxin subunit B conjugated to HRP into the same muscle (Curfs et al , 1993) The somata were located dorsolaterally in the ventral horn gray matter in C7 C8 (Fig 1A-C, 2, 3), although the rostral boundary varied from the caudal third of C6 to the rostral part of C7 In a few rats the flexor MNs were stained exclusively, without labelling of other neuronal populations The position of the MNs was constant at the three postnatal ages investigated

Labelling of interneurons in the cervical spinal cord
In addition to labelling of the MNs, most animals at all postnatal ages investigated, also showed transneuronal labelling of INs in the cervical spinal gray At the survival times used, neuronal labelling with PRV varied from only the nucleus being stained to complete filling of both the nucleus, the cytoplasm and the dendritic trees At P1 (i e rats injected at the day of birth and perfused 24 h later) only a few transneuronally labelled neurons were found These were located ipsilaterally in C5-8, dorsally in the ventral horn (Fig 1A, B, Fig 2) At P7 considerable variability in staining intensity was found Three types of infection could be discriminated lightly, intermediately, or heavily infected rats In animals with a light infection only a few INs were labelled in C4-8, located dorsally in the ventral horn, thus showing resemblance to rats at P1 (Fig 2) In heavily infected rats many INs were labelled from C1 to thoracic spinal cord segment 1 (Th1), although the majority was located in C3-C8 Most INs were found in the ipsilateral half of the spinal cord, dispersed throughout most of the dorsal and lateral side of the ventral horn, in the intermediate zone and in the ventral part of the dorsal horn In addition, few INs were found on the contralateral side of the spinal cord from C1-Th1, dispersed in the ventral horn Besides, a labelled non-neuronal population was encountered, probably representing glial cells This population was most prominent on the site of the primary infection, i e in the direct vicinity of labelled MNs in C7-8 (Fig 1C) In intermediately infected rats labelled INs were found in the ipsilateral C1-Th1, dorsally in the ventral horn and in the intermediate zone, although their number was considerably smaller than in heavily infected animals Also, only a few labelled neurons were found on

the contralateral side Virtually no non-neuronal cells were found (Fig 1D, 3) The labelling observed at P21 was consistent with that in intermediately infected rats at P7 Labelled INs were found in C1-Th1, dorsally in the ventral horn Again, only few non-neuronal cells stained positively for the PRV, located in C7-8 near the MNs (Fig 1E, F, 3)

Labelling of autonomic neurons in the thoracic spinal cord
In nearly all animals at all postnatal ages studied the intermediolateral cell nucleus (IML) in the rostral part of the thoracic spinal cord, i e Th2-5, stained positively for the presence of PRV This included labelling of many medially extending dendrites, which extended to and even across the midline However, no evidence was found of transneuronal transport to its afferent nuclei The labelling observed at all three postnatal ages investigated showed large resemblance (not shown)

Discussion

Labelling of motoneurons in the cervical spinal cord
After the PRV is taken up in the neuromuscular junction and retrogradely transported, it primarily infects the MNs in the cervical spinal cord segments C7-8, which are located in the dorsolateral part of the lateral motor column (LMC) in the ventral horn This is in perfect agreement with the labelling of MNs found after injection of cholera toxin subunit B conjugated to HRP into the flexor muscle of the distal forelimb of rats of various postnatal ages, as previously described (Curfs et al , 1993)

Upon arrival in the cytoplasm, the virus enters the nucleus and is replicated Some anterograde transport was reported for PRV to occur, however its speed is much slower than for retrograde transport (Haxhiu et al , 1993, Jansen et al , 1992), and with the relatively short survival times used in the present study it can be neglected The newly formed virus particles enter the cytoplasm again and are transported retrogradely into the dendrites

Labelling of interneurons in the cervical spinal cord
The virus membrane of the newly formed virus particles can merge with either the somatic or the dendritic membrane and the virus capsid can infect another neuron As was recently shown in electronmicroscopic studies (Card et al , 1993, Rinaman et al , 1993), the neuronal infection results in a specific reaction of glial cells, leading to the isolation of the infected cell by astrocytes and microglia Possible virus particles in the extracellular space are taken up by astrocytes, where no replication occurs Since no virus capsids were encountered in the extracellular matrix, it was concluded that transneuronal transfer occurs exclusively in the synapse This conclusion is supported by experimental evidence provided by other authors (Jansen et al , 1993, Strack and Loewy, 1990) Some caution must be taken with the interpretation of the results in the present study, since the transneuronal transfer of PRV in young, developing rats has not yet been investigated, although it most likely is comparable to the transfer observed in the adult

Transneuronal infection was noted in the present study since in most animals with MN labelling also staining of INs was encountered It is however apparent that a large variation in labelling intensity occurred This variation was not restricted to the labelling of the INs but was also found in case of the autonomic neurons and the MNs Although the underlying mechanisms of the labelling variability are to date still unknown, the phenomenon itself was likewise reported by other authors (Jansen et al , 1992, Marson et al , 1993, Schramm et al , 1993, Ter Horst et al , 1993) Possible explanations may be either small alterations in the injection sites, thereby resulting in a variable number of axons taking up the PRV or different immunological backgrounds in the rats With respect to the latter hypothesis it should be noted that interindividual variability was encountered even between littermates In spite of the variability observed in the present study it is, however, mainly manifested in the number, and to a much smaller extent in the location of PRV infected neurons, as was also noted by other authors (Marson et al , 1993) Nevertheless the results obtained in heavily infected rats are only used as indirect evidence since the possibility of aspecific labelling can not be excluded This is supported by the fact that many labelled glial elements were found in the vicinity of the labelled MNs

Consistent results were obtained in intermediately infected rats at P7 and at P21, and in lightly infected rats at P7 and at P1, respectively Based on the short survival time used in the present study, it can be assumed that from the MNs only one synapse is crossed by the PRV, thereby infecting only last-order INs In favour of this assumption is the fact that our results show striking similarity to the labelling pattern after WGA-HRP injections into the cat deltoid muscle as previously described with respect to the cervical spinal cord (Alstermark and Kummel, 1986, 1990a, b) Nevertheless, it can not be excluded that few higher-order INs also became infected

Based on their location, different IN populations can be discerned after injecting PRV into the distal forelimb flexor muscles In the same as well as the adjacent segments where labelled flexor MNs are located, i e C7-8, labelled INs were found principally in the medial and dorsal part of the ventral horn

and in the intermediate zone These INs probably represent cells mediating non-reciprocal inhibition from group I muscle afferents (intermediate zone), INs mediating inhibition and excitation from group II muscle afferents (intermediate zone and dorsal part of the ventral horn), INs mediating reciprocal inhibition from group Ia afferents (ventral horn) and INs mediating recurrent inhibition (medial part of the ventral horn) INs from the caudal part of C2 to C4 probably represent propriospinal INs and mediate excitation and inhibition from descending fibre pathways, such as the CST Contralateral INs in the ventral part of the ventral horn probably play a role in postural control (Alstermark et al , 1991, Alstermark and Kummel, 1990a, b, Sterling and Kuypers, 1968, reviewed by Jankowska, 1992)

From the constant position of the IN somata at the postnatal ages investigated, it can be concluded that the spinal IN pattern of innervation is already well established at birth This is surprising, especially since much of the IN innervation upon MNs occurs through dendrites and we have previously shown that MN dendrites increase their extension in the first postnatal week, and later on during maturation, selectively decrease their extension (Curfs et al , 1993) However, it might well be that when the numbers of the INs, their dendrites, or their synapses and the type of the latter are concerned, postnatal changes can be found The hypothesis on the number of INs can hardly be addressed with the PRV technique because of the high variability The number and extension of dendrites and number and type of synapses per neuron however, can well be established with PRV because of the complete filling of the dendrites and the compatibility of the technique with electron microscopicy (Card et al , 1993, Rinaman et al , 1993)

Labelling of autonomic neurons in the thoracic spinal cord In the thoracic spinal cord, the labelling was confined to the IML in Th2-5, with a few rare exceptions in Th1 which can be attributed to transneuronal transport from MNs In this nucleus the sympathetic preganglionic neurons (SPNs) are located The same phenomenon was observed after PRV injections into the medial gastrocnemic muscle of adult rats besides infected MNs in the lumbar spinal cord also SPNs in the IML in Th11 to lumbar spinal cord segment 2 were found (Rotto-Percelay et al , 1992) These neurons will have taken up the PRV through terminals innervating the blood vessels near the injection site and might be responsible for vasoconstriction The possibility that PRV particles enter the blood vessels and are transported to the heart, where they are taken up by terminals innervating the myocard, can be excluded SPNs in the IML innervating the heart were labelled only after injection of PRV into the myocard and not by transport from other sites (Ter Horst et al , 1993) Injections of PRV into the heart resulted in SPNs in the IML in Th1-6 being labelled (Ter Horst et al , 1993) However, the segmental overlap is likely to be coincidental, especially since SPNs innervating different organs are located intermingled in clusters in the IML (Jansen et al , 1993) No evidence was found of labelled parasympathetic preganglionic neurons, although the survival times probably were too short, with respect to the transport rate, to demonstrate these nuclei In the present study no transneuronally infected neurons terminating upon SPNs were found, although to totally exclude this possibility control experiments would be needed either in rats with a spinal transection at the level of Th1, or in rats in which the superior cervical ganglion is excised Both experiments are, however, hardly feasible in young developing rats The high affinity of the PRV for autonomic neurons explains its popularity in studying the neuronal innervation of various autonomic organs (Dobbins and Feldman, 1994, Haxhiu et al , 1993, Jansen et al , 1992, Loewy et al , 1994, Loewy and Haxhiu, 1993, Marson et al , 1993, Standish et al , 1994, Ter Horst et al , 1993, among others)

In conclusion, we have shown that spinal INs can well be labelled transneuronally after injection of PRV into the distal forelimb flexor muscle throughout postnatal maturation Based on their position several subpopulations can be discerned, propriospinal neurons in C2-C4, INs mediating excitation and inhibition from primary afferents in C5-C8, and contralateral INs When the position of INs innervating distal forelimb flexor muscles is concerned, no postnatal changes were detected However, other characteristics of INs such as their dendritic fields and/or the number and type of their synapses may show postnatal variations Although PRV has a high affinity for autonomic neurons it will not pose large problems because when short survival times are used no transneuronal transport from these autonomic nuclei seems to occur

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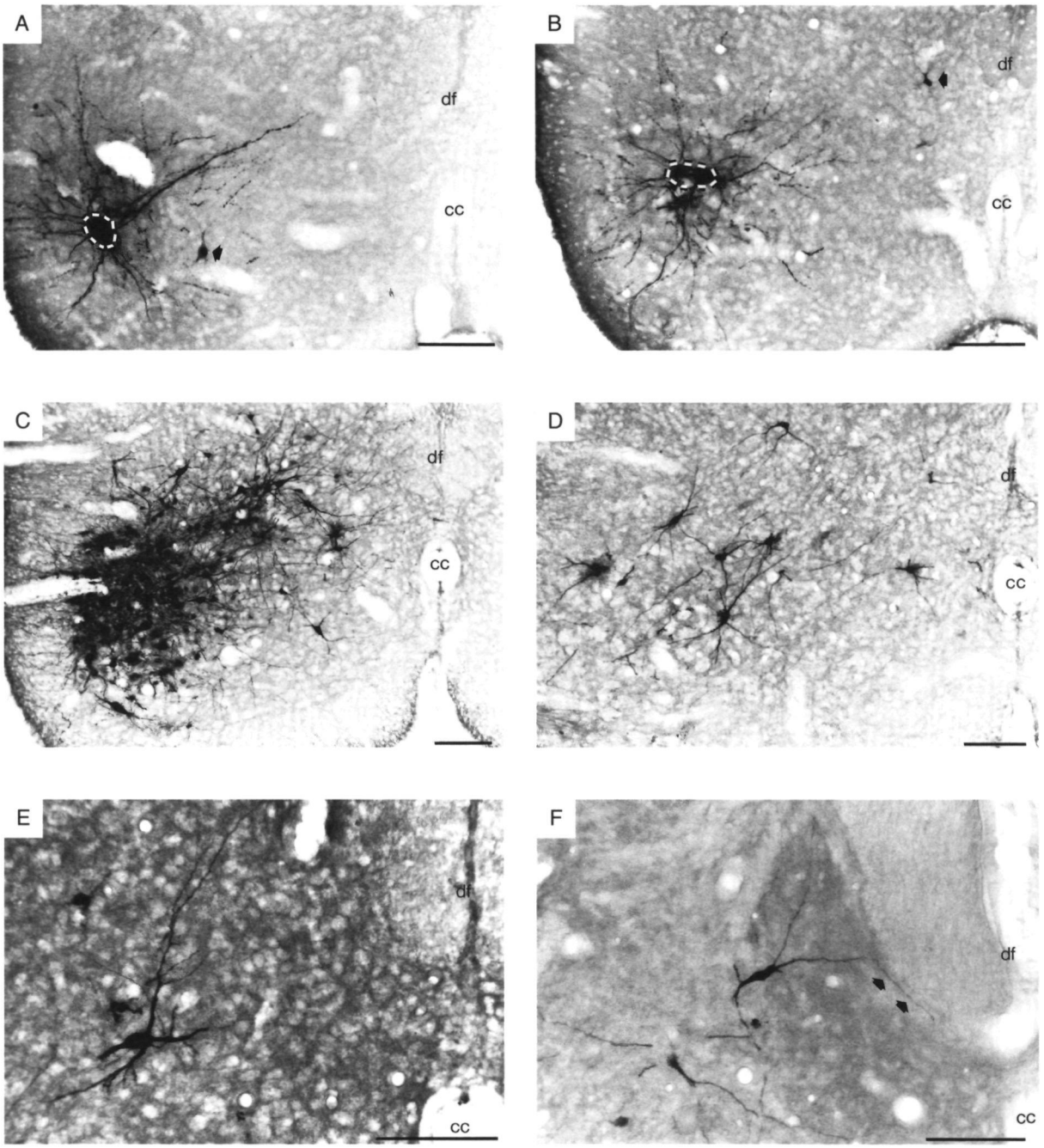

Fig. 1. Photomicrographs of transversel sections of the cervical spinal cord after pseudorabies virus injections into the flexor muscles in the distal forelimb of postnatal rats at various ages. df = dorsal funiculus, cc = central canal. The presumed motoneuron area is indicated by the dashed circle. A., B. Cervical spinal cord segment 7 at postnatal day 1. Interneurons are indicated by arrows. C. Cervical spinal cord segment 7 at postnatal day 7 of a heavily infected rat. Note the presence of many small labelled cells. D. Cervical spinal cord segment 7 at postnatal day 7 of an intermediately infected rat. Several interneurons are labelled. E. Interneuron at P21 in cervical spinal cord segment 5. F. Interneuron at P21 in cervical spinal cord segment 6. Note the dendrite marked with arrows which runs in close apposition to the dorsal funiculus. Scale bar in each photomicrograph = 100 μm.

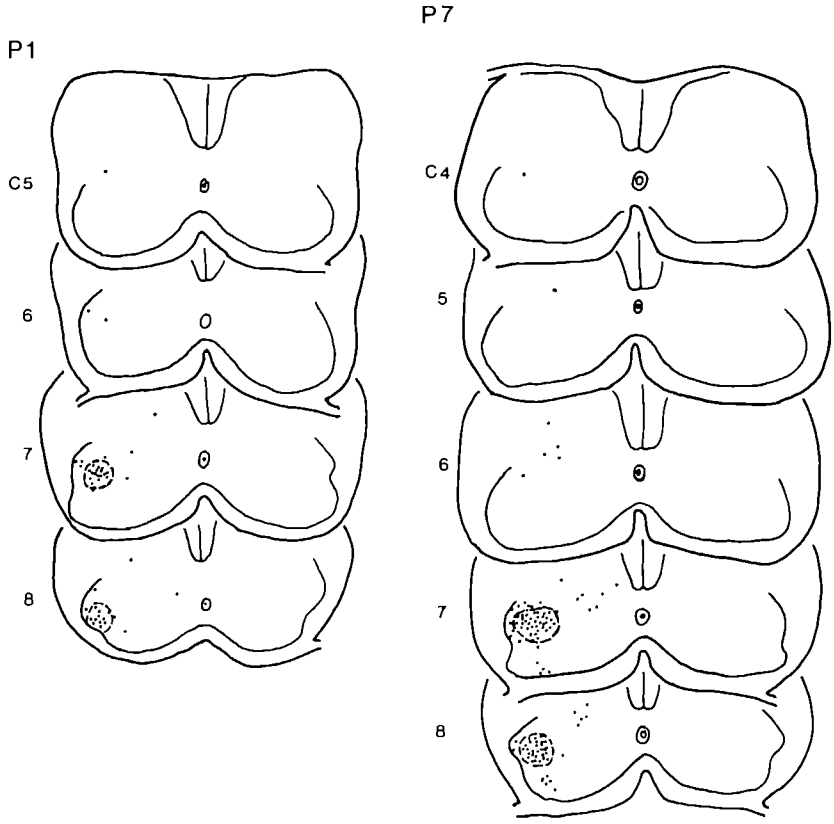

Fig. 2. Composite illustration of schematized drawings of cervical spinal cord segments 5 (C5) to C8 at postnatal day 1 (P1, left side) and of C4-C8 of a lightly infected rat at P7 (right side). Sections with labelled neurons were drawn and the data of all sections were pooled onto a representative section of their respective segment. The presumed motoneuron area is indicated by the dashed circle. It is apparent that the location of both the motoneurons and the interneurons shows large resemblance at both postnatal ages.

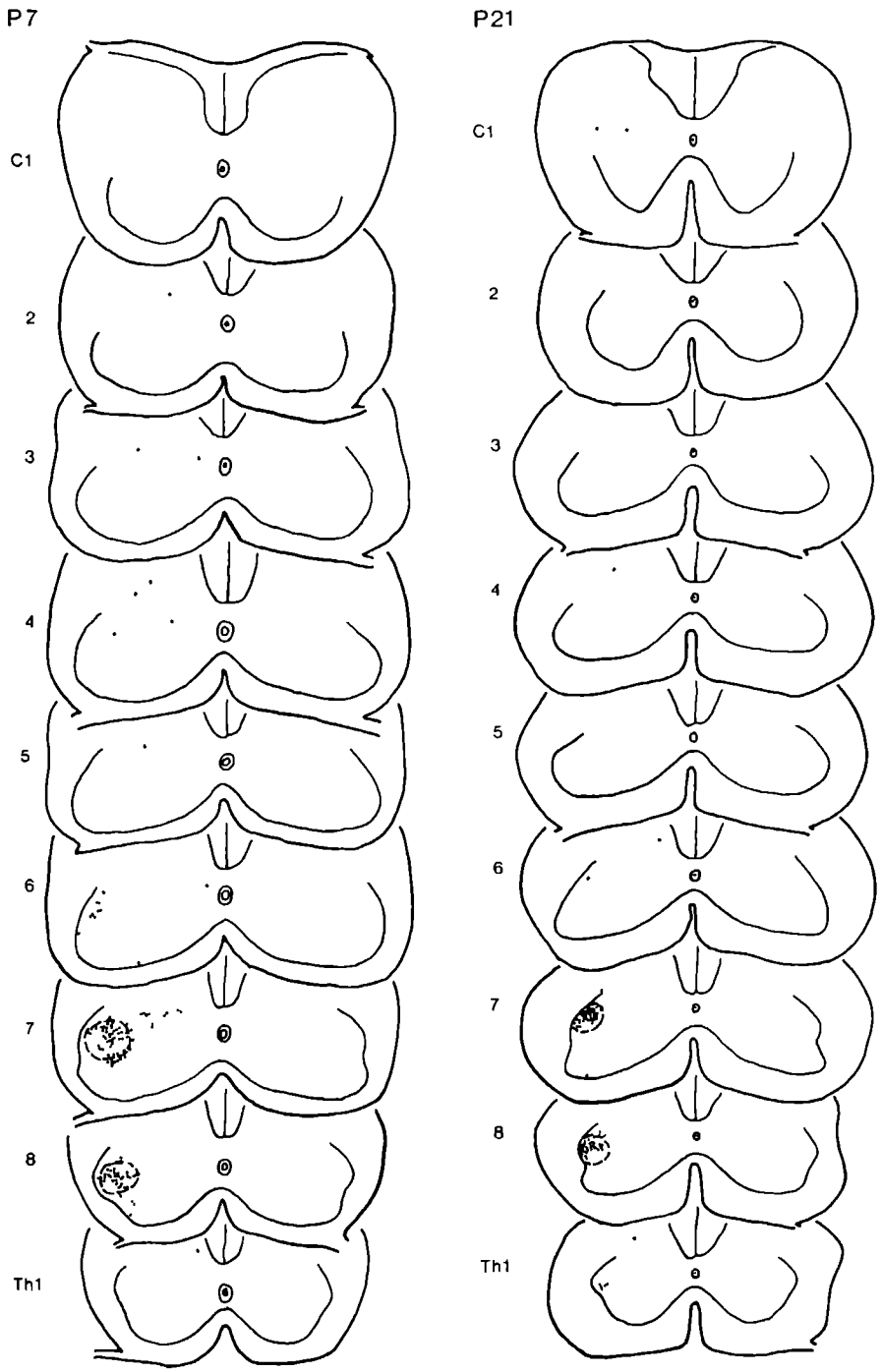

Fig 3 Composite illustration of schematized drawings of cervical spinal cord segments 1 (C1) to thoracic spinal cord segment 1 (Th1) of a intermediately infected rat at postnatal day 7 (P7, left side) and C1-Th1 at P21 (right side) Sections with labelled neurons were drawn and the data of all sections were pooled onto a representative section of their respective segment The presumed motoneuron area is indicated by the dashed circle It is apparent that the location of both the motoneurons and the interneurons shows large resemblance at both postnatal ages

57 Cervical spinal interneurons innervated by the corticospinal tract

7.1. Induction of c-fos expression in cervical spinal interneurons after kainate stimulation of the motor cortex in the rat

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Key words Corticospinal tract, Cervical spinal cord, Interneurons, c-fos, Rat

Summary

he expression of the immediate-early gene c-fos was used as a marker of neuronal activity to investigate the cervical spinal interneuron populations involved in the cortico-motoneuronal pathway Adult rats received unilateral kainate injections in the forelimb area of the primary motor cortex After a survival period of 90 min , during which the animals showed vehement twitching of the contralateral forelimb, the rats were perfused and their brains and cervical spinal cords processed for fos-like immunoreactivity In the cervical spinal cord fos-like immunoreactive neurons were found bilaterally in the dorsal horn and in the intermediate zone, though contralaterally significantly more labelled nuclei were encountered in two different areas One area closely resembles the corticospinal terminal field as demonstrated with anterograde horseradish-peroxidase tract-tracing and the other reflecting primary afferent and noxious sensory neurons in the dorsal horn Thus by monitoring the evoked expression of the immediate early gene c-fos, structural components of the rat motor system can be identified

Introduction

The corticospinal tract (CST) is a major fibre bundle originating in the cerebral cortex layer V pyramidal neurons, and projecting to the spinal cord (Armand, 1982, Stanfield, 1992) It is implicated in the control of voluntary movements, and in particular those of the fingers (Armand and Kably, 1993, Castro, 1972a, b, Castro-Alamancos and Borrell, 1993, Kalil, 1988, Montoya et al , 1991, Porter, 1987, Schrimsher and Reier, 1993, Wannier et al , 1991, Whishaw and Kolb, 1988) Animals with high digital skills (such as monkeys but also rodents such as the rat and the hamster) are characterized by a direct CST innervation of the motoneurons (MNs) in the lateral motor column of the cervical spinal cord (Armand, 1982, Curfs et al , 1994a, Elger et al , 1977, Heffner and Masterton, 1975, Kuang and Kalil, 1990, Kuypers, 1982, Liang et al , 1991, Porter, 1987) In previous investigations the maturation of MN dendritic fields in cervical spinal cord segments 7 and 8 (C7-8, Curfs et al , 1993) and the outgrowth of the CST axons into the spinal gray of these segments (Curfs et al , 1994a) has been studied From these studies it was concluded that in the adult rat the MN dendrites, especially those of the MNs innervating the long flexors of the distal forearm, and the CST terminal field show a large area of overlap In this area direct cortico-motoneuronal synaptic contacts (Elger et al , 1977, Liang et al , 1991) may be found However, the intermediate target of the CST, the interneuron population through which much of the CST influence upon MNs takes place remains to be established

After a stimulus to a neuron immediate early genes such as c-fos are transneuronally expressed in the nucleus, where these genes are then translated into proteins (Armstrong and Montminy, 1993, de Felipe et al , 1993, Morgan and Curran, 1991, Sharp, 1993), which can be visualized by immunohistochemistry The c-fos technique thus provides a powerful tool for the visualization of functionally related chains of neurons such as the corticospinal system In the present paper the motor cortex was stimulated with kainate, a potent glutamate agonist Glutamate receptors are abundant in layer V pyramidal neurons of the rat motor cortex and the CST is most likely glutamatergic (Giuffrida and Rustioni, 1989, Wisden and Seeburg, 1993) Fos-like immunoreactivity (Fos-Li) then was analyzed in the cervical spinal cord Parts of these results have been reported previously in abstract form (Curfs et al , 1994b)

Materials and methods

Animals

In the present study ten young adult (approximately 60 days old and 200 grams) Wistar rats (Central Animal Laboratory, University of Nijmegen) were used, subdivided into three groups One group of four animals was used to study the CST projection area in the cervical spinal gray with horseradish-peroxidase (HRP) anterograde tract tracing (the same material as in Curfs et al (1994a) The other six animals were used to study the interneuron population in the cervical spinal cord three rats received kainate injections in the cerebral cortex and the other three rats were used as their sham-operated controls

HRP labelling of the CST

The method for CST labelling with HRP is described in detail elsewhere (Curfs et al , 1994a) In brief, after anaesthesia with sodium pentobarbital (60 mg per kg body weight, i p), HRP (Boehringer Mannheim, grade i)-gels (Griffin et al , 1979) were implanted into the cerebral cortex encompassing the entire sensorimotor cortex After a postimplantation survival time of 48 hours the rats were reanaesthetized (90 mg sodium pentobarbital per kg body weight) and transcardially perfused After dissection from the skull and spine respectively, the brain and spinal cord were postfixed, cryoprotected and embedded in gelatin Using a freezing microtome 30 µm sections were cut in the transverse plane The sections were reacted for HRP histochemistry using the 3-step procedure tetramethylbenzidine-ammoniumheptamolybdate incubation, next diaminobenzidine-nickel (DAB-Ni) stabilization, followed by DAB-cobalt-glucose oxidase intensification, as described previously The sections were mounted onto glass slides using a gelatin-alcohol solution, counterstained with neutral red, dehydrated and coverslipped with Depex The cervical spinal cord segments 3 to 8 (C3-C8) were examined under dark field illumination Photomicrographs were made using an automatic Zeiss photomicroscope II

Fos labelling of the cervical interneuron population

In the fos experiments the anaesthesia was initiated by placing the rats in a glass box containing tissue paper saturated with ether During the operation the anaesthesia was maintained with a tube filled with a gauze saturated with ether The animals were then transferred to a stereotaxic apparatus and the skin overlying the skull was incised In one group of 3 rats small holes were drilled into the skull and 3 separate injections of kainate (100 ng in 0 5 µl per injection) were made into the forelimb area motor cortex,

stereotaxic coordinates 3 5 mm anterior of bregma and 1 mm lateral of the midline, 2 mm anterior/3 mm lateral, and 0 5 mm anterior/1 mm lateral (Neafsey et al , 1986) The wound was then sutured and the animals were allowed to recover The other group of 3 rats received a sham-operation consisting of only an incision and subsequent suturing of the skin After a 90 min survival period (which after preliminary experiments appeared to be the optimum) during which the animals showed vehement twitching of the contralateral forepaw, the rats were transcardially perfused under deep ether anaesthesia with ice-cold 0 1 M phosphate buffered saline (PBS, pH 7 4) followed by Samboni's fixative (1 8% paraformaldehyde and 7 5% picrinic acid in PBS, pH 7 5) After dissection from the skull and spine, the brain and spinal cord respectively were postfixed by immersion in the above mentioned fixative for 24 h and stored in PBS As soon as possible 50 µm vibratome sections of the brain and cervical spinal cord were cut and collected in an one in two series and processed for Fos-Li All incubations mentioned were performed at room temperature Sections were first pre-treated against endogenous peroxidase with 0 3% H_2O_2 in aqua dest, and after rinses in 0 05 M Tris buffered saline (TBS, pH 7 6), pre-incubated in 5% normal horse serum, 0 1% Triton and 0 1% BSA in TBS (TBS-BT-NHS) for 1 h Then the sections were incubated overnight in sheep IgG's against c-fos (dilution 1 2000 for brain and 1 4000 for spinal cord sections, Cambridge Research Biochemicals, batch OA 11-824) in TBS-BT-NHS After rinses in TBS the sections were incubated for 90 min in horse anti-sheep antibodies (1 100, Nordic Immunology, Tilburg) in TBS-BT, again rinsed in TBS and incubated for 90 min in sheep peroxidase-anti-peroxidase complex (1 600, Nordic Immunology, Tilburg) in TBS The sections were rinsed again in TBS and the presence of Fos-Li was visualized using a nickel intensified DAB procedure (20 mg DAB, 300 mg ammonium nickel sulphate and 10 µl 30% H_2O_2 in 100 ml 0 05 M Tris buffer, pH 7 6) for 3 min The sections were then mounted onto glass slides using a gelatin chrome-alum solution, air-dried and embedded in Depex The labelled cell nuclei in one out of four randomly selected sections were drawn under bright field illumination using a Zeiss microscope equipped with a drawing tube The spinal cord was subdivided into three parts cervical spinal cord segment 3 and 4 (C3-4), C5-6, and C7-8 The drawings of two consecutive spinal cord segments were pooled and plotted onto a representative section The number of labelled nuclei in these pooled sections were counted, and subdivisions were made for the dorsal horn, the intermediate zone, and the ventral horn Differences were tested for statistical significance by means of an ANOVA

Results

CST projection area after labelling with HRP

The CST projection area as demonstrated by HRP-gel implantation in the cerebral cortex after 48 h is shown in fig 1 From the photographs it can be deduced that labelled CST axons cover large parts of the contralateral spinal gray matter, in particular the medial portion of the dorsal horn, the entire intermediate zone and the ventral horn with exception of the lateral part of the lateral motor column The number of fibres was highest in the direct vicinity of the CST and decreased progressively further distally It can also be noted that the CST projection area increased progressively from C3-4, and C5-6 to a maximum in C7-8 with regard to both the number of labelled fibres and the size of the projection area

Fos induction after kainate injections

Approximately 20 min after the injections, the cortex stimulated animals started to display typical kainate induced behaviour, consisting of nearly constant locomotion, and misplacement and twitch-like movements of the contralateral forelimb, which was not observed in their sham-operated counterparts Examination of sections taken from their brains after a 90 min survival period revealed an increased Fos-Li in the ipsilateral cerebral cortex, including the forelimb representation area (fig 2A) Increased Fos-Li as opposed to sham-operated animals was also observed in the ipsilateral caudate nucleus, putamen, and red nucleus, and bilaterally in the thalamus (reticular, ventromedial and posterior nucleus), globus pallidus, subthalamic nuclei, tectum, and brainstem nuclei such as substantia nigra, pontine and raphe nuclei In the cervical spinal cord an increased number of Fos-Li cells was noted bilaterally after kainate injection in all segments examined (fig 2B-D, 3, 4) The majority of these neurons (62%) was found in the dorsal horn, the minority was located in the intermediate zone (15%) and in the ventral horn (23%) This distribution was the same in all cervical spinal cord segments studied, although the number of Fos-Li nuclei varied considerably rostrocaudally (fig 5) In addition to the distinct regional distribution of Fos-Li neurons, significantly more fos-positive nuclei were found in the contralateral half of the spinal cord as was noted in every spinal cord segment and in every region analyzed The difference was however most prominent in the dorsal horn and the largest in the combined C5-6 segment and, slightly smaller, in C7-8 (fig 4, 5) Fos-Li nuclei on the contralateral side are mainly found in

two areas, one located in the vicinity of the dorsal funiculus and one located in especially the medial portions of the superficial layers of the dorsal horn (fig. 4)

Discussion

In the present study it is clearly shown that stimulating the rat motor cortex with the powerful glutamate agonist kainate results in an increased number of Fos-Li neurons in the cervical spinal cord as opposed to that found in sham-operated rats. This finding is contrary to previous findings in literature (Wan et al., 1992). Although these authors noted similar labelling in forebrain and brain stem structures, they found no c-fos labelling in the spinal cord. Probably, the kainate stimulation as used in the present investigation is much more potent than the intracortical microstimulation of the motor cortex as applied in their experiments. That the c-fos labelling is principally found in the dorsal horn is in agreement with the data in literature. After an inflammatory stimulus to the spinal cord, neurons expressing immediate early genes belonging to the fos-family are mainly located in the dorsal horn and intermediate zone, whereas jun (another family of immediate early genes) positive neurons are mainly found in the ventral horn plus the superficial layer of the dorsal horn (Lanteri-Minet et al., 1993). Since the main component of the CST projects through the dorsal funiculus to the contralateral spinal cord gray matter (Armand, 1982), it was to be expected that most Fos-Li neurons were present in the contralateral gray matter as was exactly found in the present study.

On closer investigation of the numbers of Fos-Li neurons and their respective location, it became apparent that contralaterally two distinct populations could be discerned. One population was located adjacent to the dorsal funiculus in the dorsal horn and in the intermediate zone. This area shows great resemblance to the corticospinal projection area as was demonstrated by anterograde HRP tract-tracing. We have shown that the CST projection area increases progressively in the caudal direction and reaches its maximum in C7-8 with regard to both the number and extension of labelled CST fibres. This distribution highly corresponds to the number and location of the Fos-Li neurons. The other Fos-Li neuron population is principally located in the dorsal horn, i.e. the area that receives primary afferent input (Brown, 1981; Hirakawa et al., 1992; Sprague and Ha, 1964). Obviously, the kainate stimulation of the CST activates the premotor interneurons and in turn the motoneurons, causing locomotion and twitch-like movements of the forelimb and thereby results in the activation of primary afferents. The evoked c-fos expression pattern found closely resembles that of walking rats as was recently described (Jasmin et al., 1994) In addition, a second component can be discerned in the Fos-Li neuron population encountered in the dorsal horn, namely those located in the superficial layers of the dorsal horn, especially their medial parts. These neurons represent a subpopulation which is activated by noxious stimulation (Abbadie et al., 1994, Jasmin et al., 1994; Presley et al., 1990; Sugimoto et al , 1994; Tolle et al., 1994). Apparently, the kainate stimulation of the sensorimotor cortex also raises sensory sensation, either directly or indirectly.

The increased number of fos-IR neurons in the ipsilateral half of the spinal cord can be attributed to several factors. Firstly, this merely can be a side-effect from the increased locomotion in the contralateral forelimb which can be expressed in bilateral activity such as walking. Ipsilateral interneurons are then stimulated by primary afferent axons from the ipsilateral forelimb. Secondly, c-fos expression in spinal interneurons can be induced by bilateral descending tracts other than the CST (such as the rubrospinal, vestibulospinal, and reticulospinal tracts) originating in areas which are stimulated bilaterally by the cerebral cortex (e.g. the thalamus and brainstem nuclei). And thirdly, CST fibres in the ipsilateral spinal cord might add to the increased number of Fos-Li neurons. These might be the contralateral CST fibres which return to the ipsilateral side in the spinal cord or the minor uncrossed CST component located in the ventral funiculus (Joosten et al., 1992). Evidence for the latter hypothesis was found in one animal in which significant increased numbers of Fos-Li neurons were found located near the ipsilateral ventral funiculus.

In conclusion, in the present study we have clearly shown, that kainate injections into the cerebral motor cortex result in many spinal interneurons being labelled for the immediate early gene c-fos. At least part of these neurons are under the direct influence of the CST while others could be activated by other descending tracts or primary afferents It is also conceivable that neurons, and especially interneurons, are multiply innervated by several systems. On the other hand, only a certain subpopulation of interneurons can be labelled with the c-fos technique, since excitation is a prerequisite for the induction of immediate early genes (Armstrong and Montminy, 1993; de Felipe et al., 1993; Morgan and Curran, 1991; Sharp, 1993). It is known from the literature that the influence of the CST upon spinal neurons is not only excitatory, but also inhibitory (Alstermark and Sasaki, 1985; Cheney et al , 1985, Flament et al., 1992;

Illert and Wiedemann, 1984). Nevertheless, the induction of c-fos after kainate stimulation of the cerebral cortex provides a powerful tool in the study of the influence of the CST upon the spinal cord and future research should be aimed at the elucidation of the influence of other structures upon the corticospinal system, for instance by lesion of other descending tracts and/or the primary afferents

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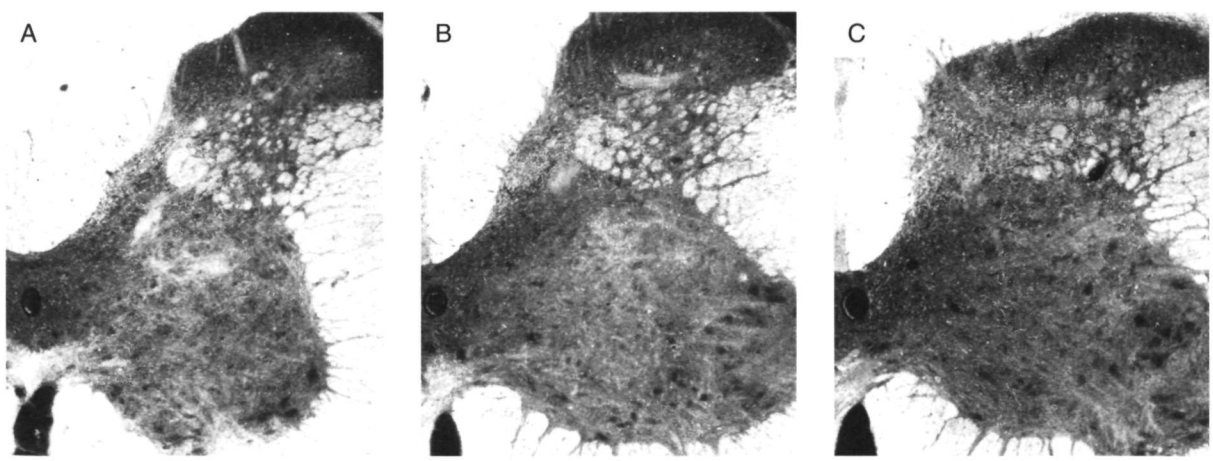

Fig. 1. Photomicrographs of the contralateral corticospinal tract and spinal gray matter in cervical spinal cord segment 3 (A), 5 (B), and 7 (C) 48 h. after HRP-gel implantation into the cerebral motor cortex under dark field illumination. Scale bar = 200 μm.

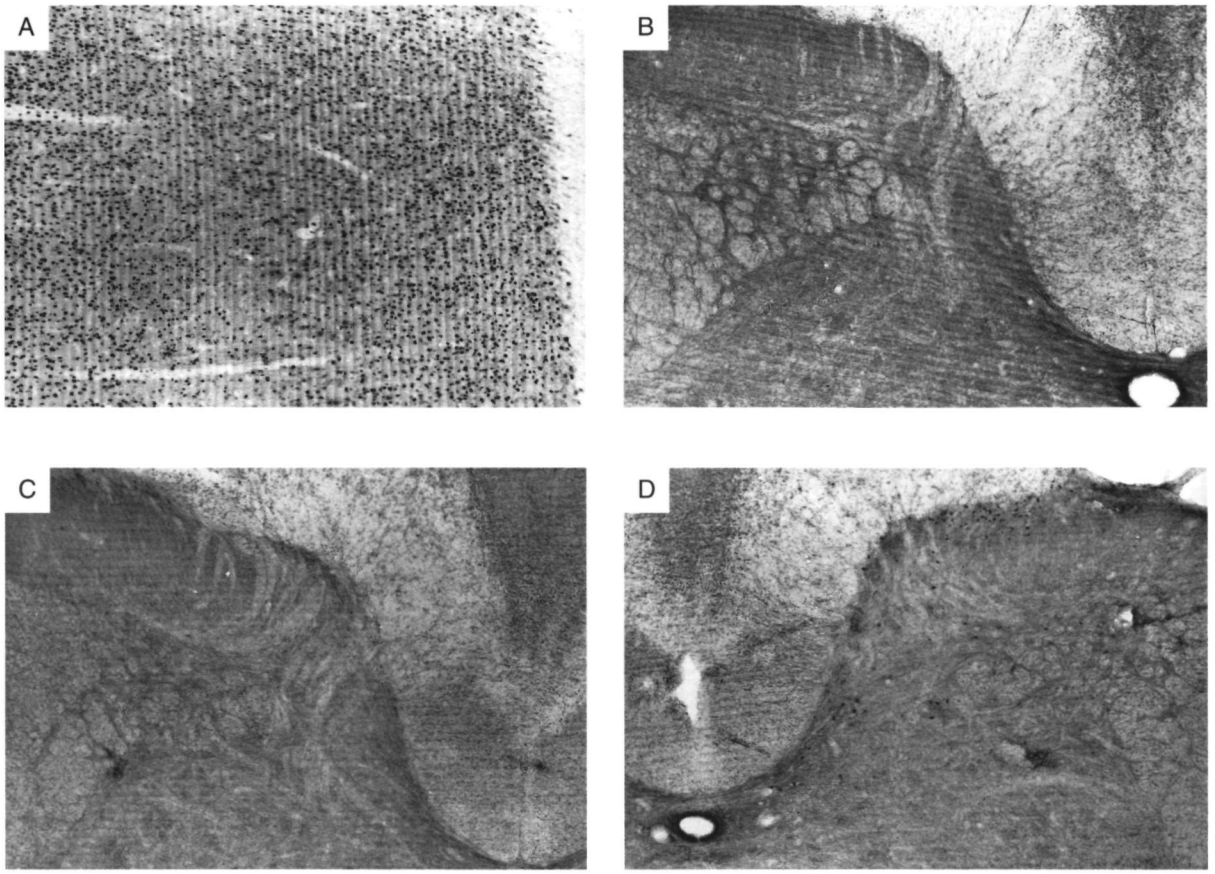

Fig. 2. (A) Photomicrograph of the Fos-Li in the ipsilateral cerebral cortex 90 min. after kainate injections into the cerebral motor cortex. Pia mater is to the right. Note that c-fos is expressed in nearly all neurons. (B) Photomicrograph of the Fos-Li in the dorsal horn and intermediate zone in cervical spinal cord segments 5 to 6 (C5-6) of a sham-operated rat. Virtually no neurons are labelled. (C) Photomicrograph of the Fos-Li in the ipsilateral dorsal horn and intermediate zone in C5-6 90 min. after kainate injections into the cerebral motor cortex. Several fos-positive nuclei can be found in especially the dorsal horn. (D) Photomicrograph of the Fos-Li in the contralateral dorsal horn and intermediate zone in C5-6 90 min. after kainate injections into the cerebral motor cortex. A substantial increase in the number of fos-positive neurons as opposed to both sham-operated animals (B) and the ipsilateral half of the spinal cord of kainate injected rats (C) is found. Scale bar = 200 μm.

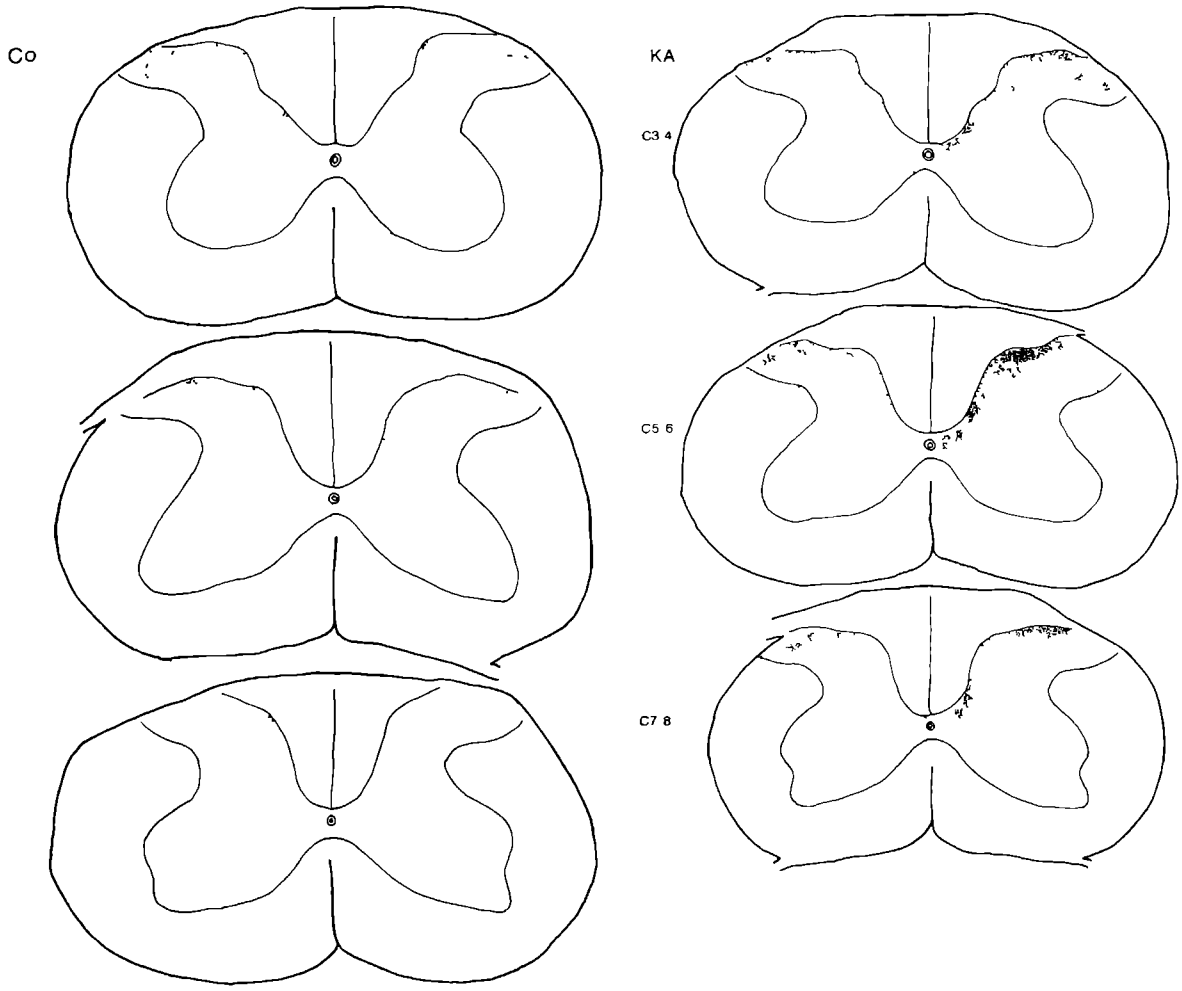

Fig 3 (left side) Composite illustration of schematized drawings of the Fos-Li in the combined cervical spinal cord segments 3 and 4 (C3-4), C5-6, and C7-8 of a sham-operated rat One in four sections were pooled and plotted onto its respective representative drawing A few c-fos positive neurons are found scattered throughout especially the dorsal horn, and no difference is noted between the left and right half of the spinal cord

Fig 4 (right side) Composite illustration of schematized drawings of the Fos-Li in the combined cervical spinal cord segments 3 and 4 (C3-4), C5-6, and C7-8 90 min after kainate injections into the cerebral motor cortex One in four sections were pooled and plotted onto its respective representative drawing A large increase is noted in the number of fos-positive neurons both on the ipsilateral (left) and contralateral (right) half as opposed to sham-operated animals It should further be noted that substantially more fos-positive neurons are found contralaterally than ipsilaterally condensed in two regions in the vicinity of the cortico-spinal tract and in the dorsal horn, especially in the medial part of the superficial layers

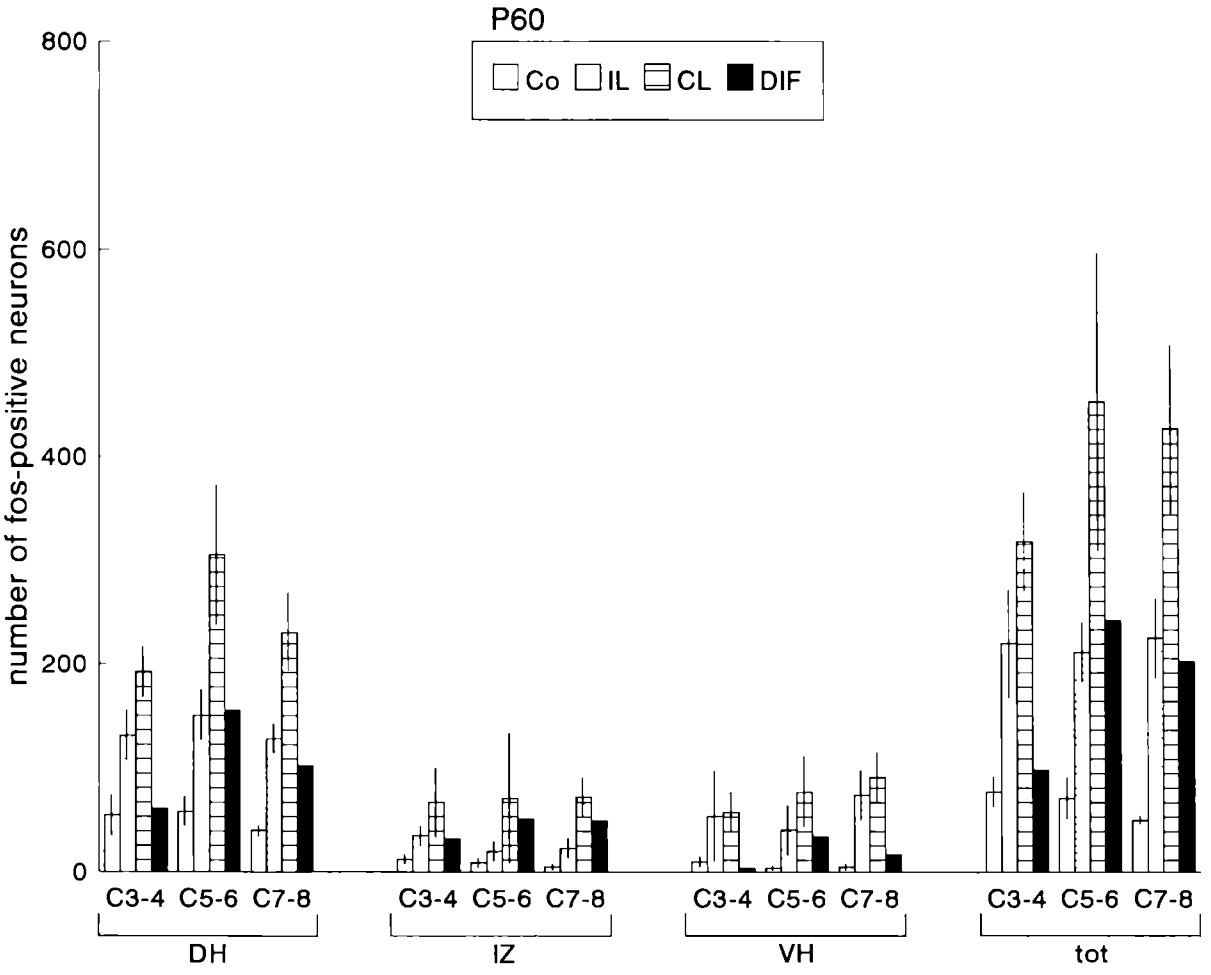

Fig 5 Histogram showing the mean numbers and standard deviation of Fos-Li neurons in sham-operated (Co) and motor cortex kainate injected adult (P60) rats in the combined cervical spinal cord segments 3 and 4 (C3-4), C5-6, and C7-8 For kainate injected animals a further differentiation is made for the ipsilateral (IL) and contralateral (CL) side and the difference between these two sides is represented by the filled bars (DIF) The spinal cord is subdivided in dorsal horn (DH), intermediate zone (IZ), and ventral horn (VH), total numbers (tot) are also shown From this figure it can be concluded that kainate injections result in more neurons being labelled for c-fos, and that the increase is largest in the dorsal horn, in the combined C5-6, and on the contralateral side

7.2. Transient functional connections between the developing corticospinal tract and cervical spinal interneurons as demonstrated by c-fos immunohistochemistry

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Summary

The corticospinal tract is the longest fibre pathway in mammals, originating in the cerebral motor cortex and projecting to the spinal cord In rodents, its spinal projection develops postnatally making it accessible for experimental research Previous research revealed that some corticospinal axons grow transiently into the spinal gray matter and are subsequently eliminated In the present study the question was addressed whether these fibres also form transient functional connections Rats aged 14 and 60 days postnatally received unilateral injections of the potent glutamate agonist kainate into the cerebral motor cortex After a survival period of 90 min the rats were perfused and their brains and spinal cords processed for the immediate early gene c-fos by immunohistochemistry Increased levels of c-fos as opposed to sham-operated animals were observed ipsilaterally in the cerebral cortex, caudate nucleus, putamen, and red nucleus, and bilaterally in the thalamus (reticular, ventromedial and posterior nucleus), globus pallidus, subthalamic nuclei, tectum, and brainstem nuclei such as substantia nigra, pontine and raphe nuclei In the cervical spinal cord most c-fos immunoreactive neurons were found contralaterally in the presumed corticospinal tract projection area and in the superficial layers of the dorsal horn, the latter probably reflecting primary afferent and noxious sensory neurons Ipsilaterally, increased c-fos immunoreactivity was found, most likely being caused by ipsilaterally projecting corticospinal axons as well as walking Most c-fos positive neurons were located in cervical spinal cord segments 5 and 6 and slightly less in cervical spinal cord segments 7 and 8 During maturation a decrease was observed in the number of c-fos positive neurons which was especially obvious in the corticospinal tract projection area This decrease shows high resemblance with the transient corticospinal projections observed previously using anterograde tract-tracing (Curfs et al , 1994a) It is concluded that during development corticospinal axons form transient functional connections with interneurons and that this part of corticospinal tract maturation shows resemblance with peripheral nervous system development

Introduction

During development, neurites are capable of forming correct functional contacts with their target after

growing out over sometimes long trajectories Immature central neurons even have the plasticity of regrowing past a lesion-site and of re-establishing their correct projection pathway including correct synaptic contacts, with no loss of functionality In contrast, sometime during development this capacity is lost, resulting in loss of functionality after a lesion (Armand and Kably, 1993, Castro, 1972a, b, Kalil, 1988, Kuang and Kalil, 1990, Martin and Xu, 1988) Therefore, understanding of the underlying developmental events might provide insight in how functional recovery can be achieved in the adult after damage of the central pathways

The corticospinal tract (CST) is the longest fibre pathway, originating in layer V pyramidal neurons of the cerebral motor cortex and projecting to the spinal cord (Armand, 1982, Stanfield, 1992, among others) It plays a role in the control of the fine, volitional digital movements, especially flexion of the fingers (Armand and Kably, 1993, Castro, 1972a, b, Castro-Alamancos and Borrell, 1993, Kalil, 1988, Montoya et al , 1991, Porter, 1987, Schrimsher and Reier, 1993, Wannier et al , 1991, Whishaw and Kolb, 1988) The rodent CST is used as a model in developmental studies since the outgrowth of CST axons throughout the spinal cord white matter, their entrance into the spinal gray and subsequent synaptogenesis occur postnatally making it accessible for experimental manipulations (Curfs et al , 1994a, de Kort et al , 1985, Donatelle, 1977, Gorgels et al , 1989, Gribnau et al , 1986, Joosten and Gribnau, 1989, Joosten et al , 1987, O'Leary and Terashima, 1988, Reh and Kalil, 1981, Schreyer and Jones, 1982, Stanfield, 1992) Using anterograde tract-tracing we have previously shown that in the rat during the outgrowth of the CST into the spinal gray overgrowth of the target occurs, i e CST fibres grow past their target and later during development the corticospinal projection is fine-tuned by axon elimination (Curfs et al , 1994a) In the present investigation, we analyzed whether these transient fibres also form functional connections which, consequently, also disappear later in development

Immediate early genes such as c-fos are expressed in the nucleus of a neuron after an excitatory stimulus to that neuron (Armstrong and Montminy, 1993, de Felipe et al , 1993, Morgan and Curran, 1991, Sharp, 1993) These genes are then translated into proteins which can be visualized by immunohistochemistry This technique is now well established and can be used to demonstrate functionally connected chains of neurons In the present study the c-fos technique was used to study the maturation of the CST projection on the interneuron population in the cervical spinal cord by injecting the potent glutamatergic agonist kainate into the cerebral motor cortex Glutamate receptors are abundant in the rat motor cortex and the CST is most likely glutamatergic (Giuffrida and Rustioni, 1989, Wisden and Seeburg, 1993) Rats at postnatal day 14 (P14) and young adult rats at P60 were used Based on the CST projection field which was maximal at P10 and at P14 just started to decrease (Curfs et al , 1994a), and on preliminary ultrastructural studies where it was concluded that there is a time-lag between the arrival of CST fibres and the formation of synapses (personal observation) it was expected that at P14 maximal or near to maximal functional contacts could be found The results described here were previously published in abstract form (Curfs et al , 1994b)

Materials and methods

Animals

In the present study 12 Wistar rats (Central Animal Laboratory, University of Nijmegen) were used The day of birth was designated as postnatal day 0 (P0), 6 animals were used at P14 (approximately 30-35 grams) and 6 animals at P60 (approximately 200 grams) Each age group was further subdivided into two groups three rats received kainate injections in the cerebral cortex and the other three rats were used as their sham-operated controls

Fos labelling of the cervical interneuron population

Anaesthesia was initiated by placing the rats in a glass box containing ether saturated tissue paper for 45 s The animals were then transferred to a stereotaxic apparatus During the operation the anaesthesia was maintained with a tube filled with an ether saturated gauze After incision of the skin overlying the skull, in 3 rats of both age groups small holes were drilled into the skull and 3 separate injections of kainate (100 ng in 0 5 µl per injection) were made into the forelimb area motor cortex (for P60 the following stereotaxic coordinates were used 3 5 mm anterior of bregma and 1 mm lateral of the midline, 2 mm anterior/3 mm lateral, and 0 5 mm anterior/1 mm lateral, Neafsey et al, 1986, and for P14 these coordinates were adapted to the smaller size of the brain) The wound was then sutured and the animals were allowed to recover The other 3 rats of both age groups underwent a sham-operation consisting of only an incision and subsequent suturing of the skin During preliminary experiments the optimal survival time was assessed to be 90 min After this survival period, the rats were transcardially perfused under deep ether anaesthesia with ice-cold 0 1 M phosphate buffered saline (PBS, pH 7 4) followed by Samboni's fixative (1 8% paraformaldehyde and 7 5% picrinic acid in PBS, pH

7 5) The brain and spinal cord were dissected from the skull and spine respectively, postfixed by immersion in the above mentioned fixative for 24 h and stored in PBS

As soon as possible 50 µm vibratome sections of the brain and cervical spinal cord were cut and collected in an one out of two sections series and processed for Fos-Li All incubations mentioned were performed at room temperature After pre-treatment against endogenous peroxidase with 0 3% H_2O_2 in aqua dest, and three rinses in 0 05 M Tris buffered saline (TBS, pH 7 6), the sections were pre-incubated in 5% normal horse serum, 0 1% Triton and 0 1% BSA in TBS (TBS-BT-NHS) for 1 h Then the sections were incubated overnight in sheep IgG's against c-fos (dilution 1 2000 for brain and 1 4000 for spinal cord sections, Cambridge Research Biochemicals, batch OA 11-824) in TBS-BT-NHS After three
68 rinses in TBS the sections were incubated for 90 min in horse anti-sheep antibodies (1 100, Nordic Immunology, Tilburg) in TBS-BT, again rinsed in TBS and incubated for 90 min in sheep peroxidase-anti-peroxidase complex (1 600, Nordic Immunology, Tilburg) in TBS The sections were rinsed again in TBS and then the presence of Fos-Li was visualized by incubation for 3 min using a nickel intensified DAB procedure (20 mg DAB, 300 mg ammonium nickel sulphate and 10 µl 30% H_2O_2 in 100 ml 0 05 M Tris buffer, pH 7 4) After final rinses in TBS, the sections were mounted onto glass slides using a gelatin chrome-alum solution, air-dried and embedded in Depex The labelled cell nuclei in one out of four randomly selected sections were drawn using a Zeiss microscope equipped with a drawing tube In order to visualize the results in a convenient way, the spinal cord was subdivided into three subsequent parts cervical segment 3 and 4 (C3-4), C5-6, and C7-8 The drawings of each of these two consecutive spinal cord segments were pooled and plotted onto a representative section The number of labelled nuclei in these pooled sections were then counted, in which the dorsal horn, the intermediate zone, and the ventral horn were discriminated Differences in the numbers found were tested for their statistical significance by means of an ANOVA test

Results

It was apparent that all rats receiving kainate injections into the cerebral motor cortex, irrespective of age, started to display motor behaviour after recovering from anaesthesia which was not apparent in their sham-operated counterparts after 20 min In particular, nearly constant locomotion, and twitch-like movements and misplacement of the contralateral forelimb were observed When sections from the ipsilateral cerebral motor cortex after a 90 min survival period and processed for c-fos like immunoreactivity (Fos-Li) were examined, a large increase in the number of Fos-Li nuclei was observed at both ages as opposed to sham-operated rats Furthermore, in the forelimb motor representation area virtually all neurons stained positively for this immediate early gene (Fig 1A, B) An increased number of Fos-Li nuclei as opposed to sham-operated animals was also observed in the ipsilateral caudate nucleus, putamen, and red nucleus, and bilaterally in the thalamus (reticular, ventromedial and posterior nucleus), globus pallidus, subthalamic nuclei, tectum, and brainstem nuclei such as substantia nigra, pontine and raphe nuclei Quantification of the number of Fos-Li neurons in brain structures was beyond the scope of the present investigation

Sections taken from the cervical spinal cord of sham-operated animals revealed a low number of neurons staining positively for c-fos (P14 mean total number of 53 nuclei, and P60 66 nuclei) The majority (74%) of these Fos-Li nuclei was located in the dorsal horn and the rest was equally divided between the intermediate zone and the ventral horn (Fig 1C, D, 2, 3, 4) The difference noted between the sham-operated P14 and P60 animals was statistically not significant

Kainate injections in the cerebral motor cortex resulted in a marked increase in the number of Fos-Li neurons in the cervical spinal cord in all segments and regions examined at the two postnatal ages examined (Fig 1E-F, 2, 3, 4) This increase was noted both in the ipsilateral and the contralateral half of the cervical spinal cord, although the increase was largest in the latter (P14, ipsilateral side mean total number of 310 Fos-Li neurons, and contralateral side 490 neurons, P60, ipsilateral side 219 neurons, and contralateral side 399 neurons) Again, most Fos-Li nuclei were found in the dorsal horn (69%) and the rest dispersed in the intermediate zone and the ventral horn In addition to this regional variation, the distribution along the rostrocaudal axis of the cervical spinal cord also varied considerably Most Fos-Li neurons were found in cervical spinal cord segments 5 and 6 (C5-6), and slightly less in C7-8, irrespective of age

The most important finding was the significant decrease of the number of Fos-Li nuclei in the cervical spinal cord after kainate stimulation of the motor cortex in the young adult The relative decrease was approximately 28% on the ipsilateral side and 19% on the contralateral side, although again variation along the rostrocaudal axis occurred, the relative decrease being largest in C3-4 and C5-6 (Fig 4) Fos-Li neurons in the ipsilateral half of the cervical spinal cord

were located more or less scattered throughout the spinal gray (Fig 2, 3), whereas those in the contralateral half of the cervical cord could be subdivided in two separate populations, although some overlap did occur One population was located in the vicinity of the corticospinal tract in the dorsal funiculus in the dorsal horn and the intermediate zone Apparently, especially this population decreased in size during postnatal maturation The other population was found in the dorsal horn and especially its medial part (Fig 2, 3)

Discussion

It is apparent from the present study that stimulating the rat motor cortex with kainate at postnatal day 14 and in the young adult results in many cervical spinal neurons being labelled for the immediate early gene c-fos This is in contradistinction to a previous report in which the motor cortex of adult rats was stimulated by intracortical microstimulation and no spinal neurons were found being labelled for c-fos, especially since the labelling of the forebrain and brainstem nuclei in both studies shows great resemblance (Wan et al , 1992) Probably, kainate delivers a more massive excitation of the cerebral motor cortex and as a consequence more cortical neurons are stimulated

Most Fos-Li neurons were encountered in the dorsal horn and the intermediate zone This agrees well with a recent study in which it was shown that after an inflammatory stimulus to the spinal cord, immediate early genes belonging to the fos-family are found in the dorsal horn and the intermediate zone whereas the jun-family is preferentially found in the ventral horn and the superficial layer of the dorsal horn (Lanteri-Minet et al , 1993) Since the main CST projects through the contralateral dorsal funiculus (Armand, 1982) and based upon the CST terminal field (Curfs et al , 1994a), it is apparent that in the present study the pattern of Fos-Li neurons in the cervical spinal cord can only partially be accounted for by direct corticospinal functional contacts This needs to be further elucidated before the development of the cervical spinal interneurons which are part of the corticospinal system can be discussed

Contralaterally, in the more superficial layers of the dorsal horn and especially in their medial parts, a population of Fos-Li neurons was encountered This population can be explained by two different systems of sensory input Firstly, the stimulation of the CST results in the motoneurons being excited, either directly or via interneurons This causes locomotion and twitching of the forelimb and thereby the activation of primary afferents and the induction of c-fos in the dorsal horn in areas which receive the primary afferent projection (Brown, 1981, Hirakawa et al , 1992, Sprague and Ha, 1964) The resemblance of the population of Fos-Li neurons which lie scattered throughout the dorsal horn with the c-fos expression in adult walking rats as was recently described (Jasmin et al , 1994), is striking The primary afferents can also be responsible for part of the Fos-Li neurons found in the ipsilateral dorsal horn since walking is a bilateral activity Secondly, the population of Fos-Li neurons in the medial parts of the dorsal horn closely resembles that encountered after noxious stimulation (Abbadie et al , 1994, Jasmin et al , 1994, Presley et al , 1990, Sugimoto et al , 1994, Tolle et al , 1994) The Fos-Li neurons in the ipsilateral spinal gray can be attributed to, besides the above mentioned primary afferents, to two other systems Other bilateral descending tracts than the CST (such as the rubrospinal, vestibulospinal, and reticulospinal tracts) originating in areas which are stimulated bilaterally by the cerebral cortex (e g the thalamus and brainstem nuclei) which also project upon spinal interneurons And finally, CST axons in the ipsilateral spinal cord might add to the increased number of Fos-Li neurons These might be either contralateral CST fibres which return to the ipsilateral side in the spinal cord or the minor uncrossed CST component located in the ventral funiculus (Joosten et al , 1992)

So far, age-related differences have not been discussed However, a statistical significant overall decrease of approximately 19% on the contralateral side and 28% on the ipsilateral side was observed between P14 and P60 This decrease is especially obvious contralaterally in the area of the presumed corticospinal projection, i e in the direct vicinity of the CST in the dorsal funiculus and extending laterally into the ventral part of the dorsal horn and intermediate zone It is obvious that this area is larger at P14 and that at this age more neurons stain positively for c-fos in this region This correlates well with the previously described CST projection pattern and number of CST axons in the spinal gray (Curfs et al , 1994a) which also decreases in size from P14 onwards From these results it can be concluded that developing corticospinal fibres form transient functional contacts with interneurons It can further be hypothesized that in regions where most CST fibres are eliminated also most transient functional contacts are found as demonstrated by the largest decrease of the number of Fos-Li neurons This is supported by the fact that the decrease of Fos-Li neurons is smallest in C7 8, i e the segments where most CST fibres persist into adulthood and vice versa, in C3-4 and C5-6 most fibres are eliminated and the decrease of Fos-Li neurons is largest Nevertheless, the observed decrease in the number of Fos-Li neurons can not be

addressed entirely to the developing CST, especially not in the ipsilateral half of the cervical spinal cord. Although it can be expected that in the ipsilateral side transient CST axons are present it can be speculated that transient aberrant projections in higher brain nuclei are responsible for the relatively larger decrease observed in the ipsilateral cord.

In conclusion, we have provided strong evidence that at least part of the developing corticospinal axons form transient functional connections with interneurons in the cervical spinal cord. The same phenomenon is observed in the development of the motor endplate. It is generally agreed upon that during development several motor axons form synapses upon one motor endplate. Eventually all aberrant axons are eliminated by competition for a trophic substance, which is secreted by the target in an activity-dependent manner and retrogradely transported to the motoneuron resulting in eventually only one motor axon innervating the motor endplate (Bennett and Lavidis, 1984; Brown and Booth, 1983; Brown et al., 1976; Brushart, 1993; Caroni and Becker, 1992; Henderson et al., 1993; Kalb and Hockfeld, 1992; Lichtman and Balice-Gordon, 1990; Navarette and Vrbová, 1993). Future in vitro or quantitative ultrastructural research may reveal whether the same mechanisms also apply to the developing CST.

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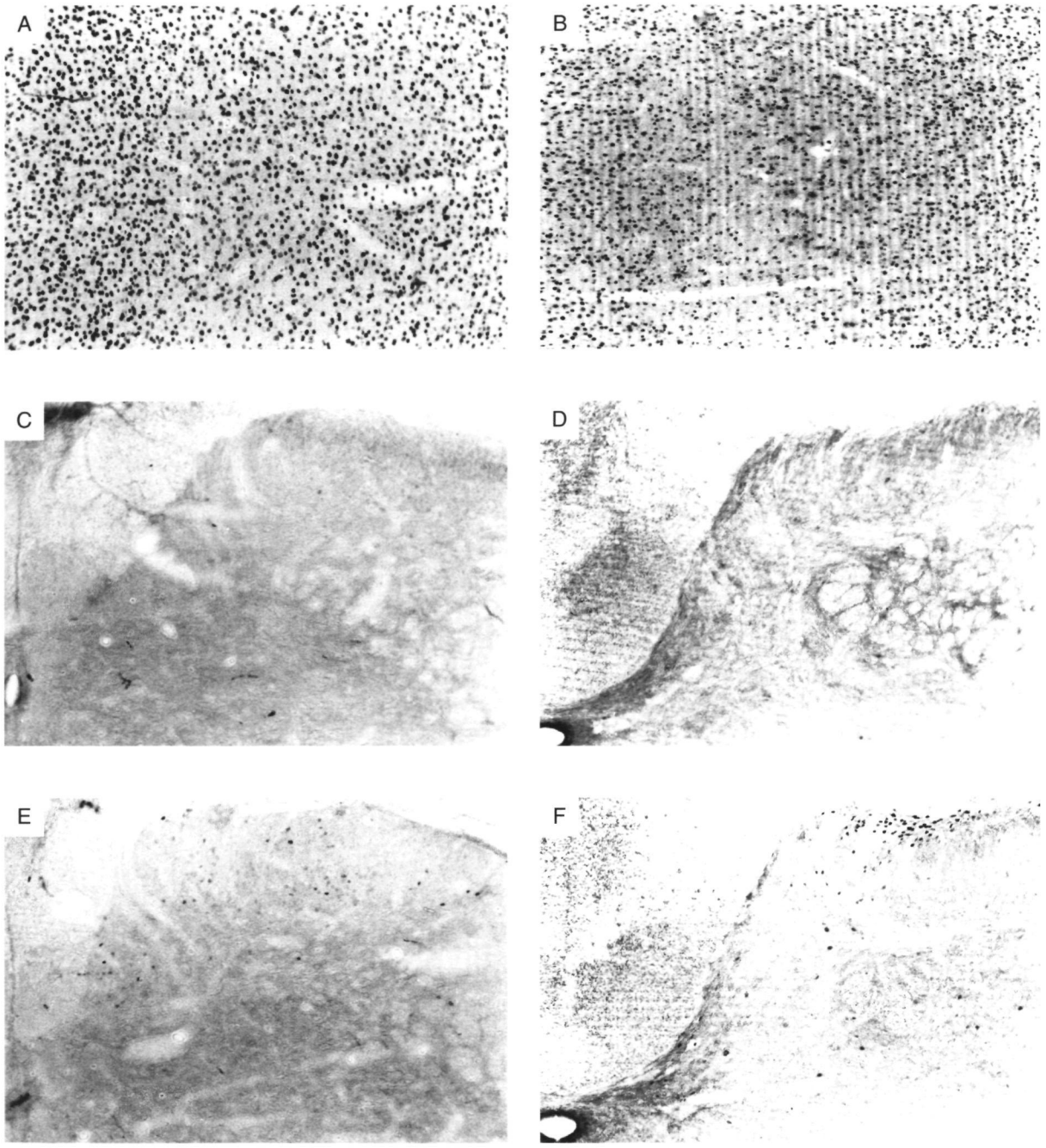

Fig. 1. Photomicrographs of 50 μm transverse sections from the ipsilateral cerebral motor cortex (A, B) and cervical spinal cord (C-F) at postnatal day 14 (A, C, E) and postnatal day 60 (B, D, F). After kainate injections into the cerebral motor cortex nearly all nuclei in the ipsilateral motor cortex stain positively for the immediate early gene c-fos. No changes were observed between P14 (A) and P60 (B). Pia mater is to the left. In the cervical spinal cord of sham-operated animals only few nuclei stained positively for c-fos at both P14 (C) and P60 (D). After kainate stimulation the number of Fos-Li neurons was significantly increased in the contralateral cervical spinal cord. This number was significantly higher at P14 (E) than at P60 (F). Scale bar = 100 μm for both P14 (left bar) and P60 (right bar).

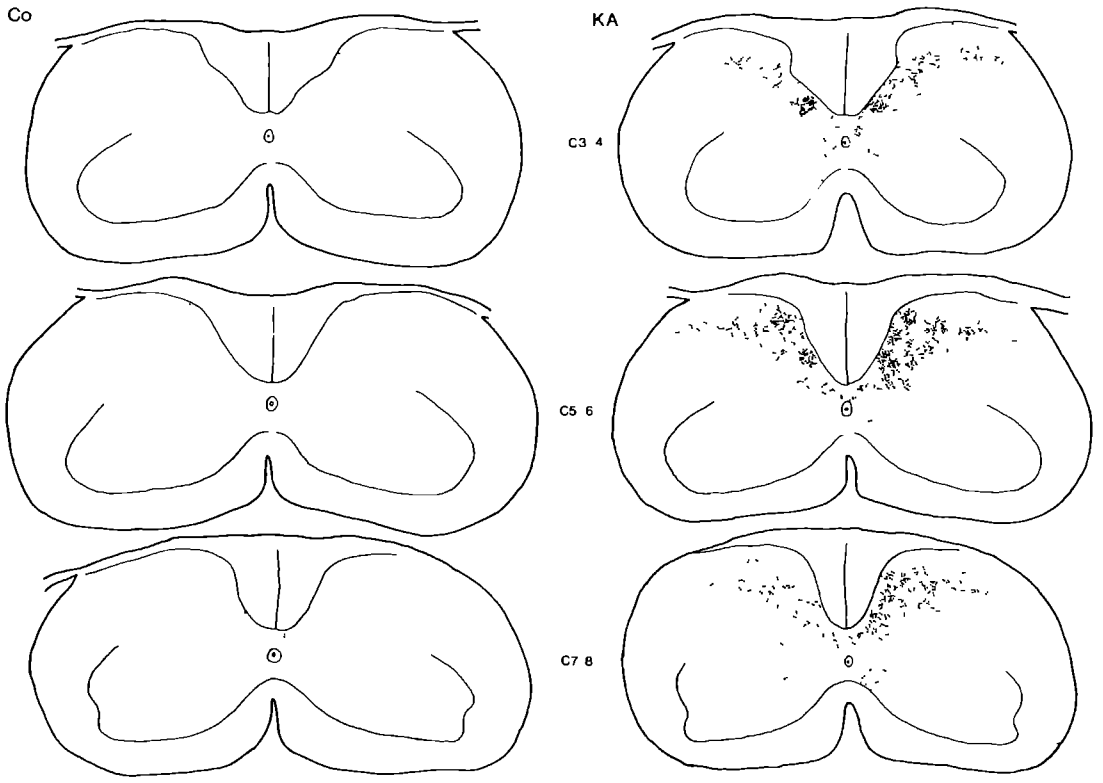

Fig. 2. Composite drawings of the Fos-Li nuclei in one in four pooled sections from the combined cervical spinal cord segments 3 and 4 (C3-4), C5-6, and C7-8 at P14 of a sham-operated rat (Co, left panel) and after kainate injections into the cerebral motor cortex (KA, right panel). When compared to sham-operated rats, a large increase of Fos-Li neurons was noted in the kainate stimulated rat, both ipsilaterally (left side of the spinal cord) and contralaterally (right side) although the increase was largest in the latter.

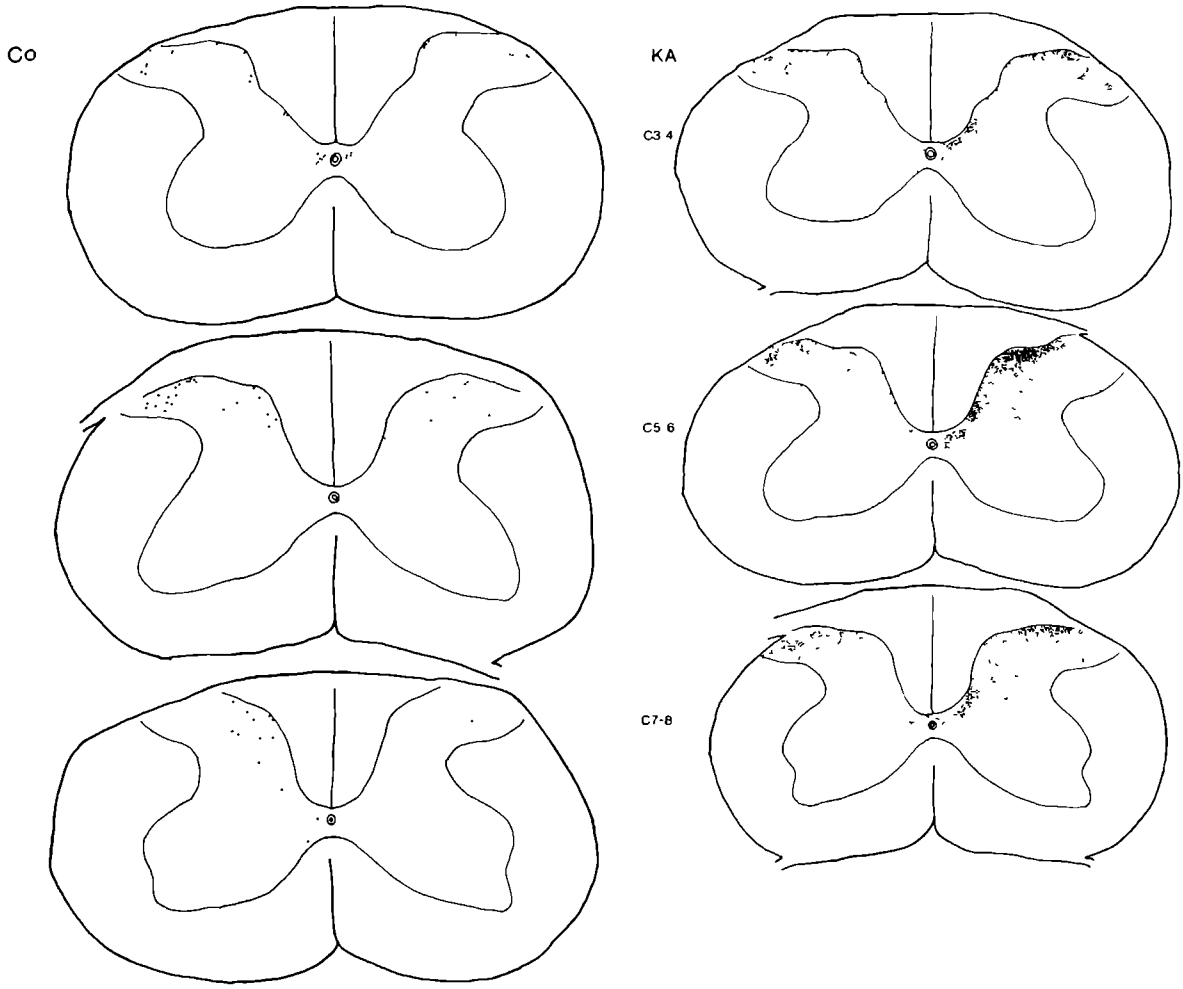

Fig. 3. Composite drawings of the Fos-Li nuclei in one in four pooled sections from the combined cervical spinal cord segments 3 and 4 (C3-4), C5-6, and C7-8 at P60 of a sham-operated rat (Co, left panel) and after kainate injections into the cerebral motor cortex (KA, right panel). When compared to sham-operated rats, a large increase of Fos-Li neurons was noted in the kainate stimulated rat, both ipsilaterally (left side of the spinal cord) and contralaterally (right side) although the increase was largest in the latter. When compared to P14 substantially less Fos-Li nuclei were encountered on both sides. Contralaterally Fos-Li nuclei were found in two regions: in the direct vicinity of the corticospinal tract and in the dorsal horn, especially in the medial part of the superficial layers.

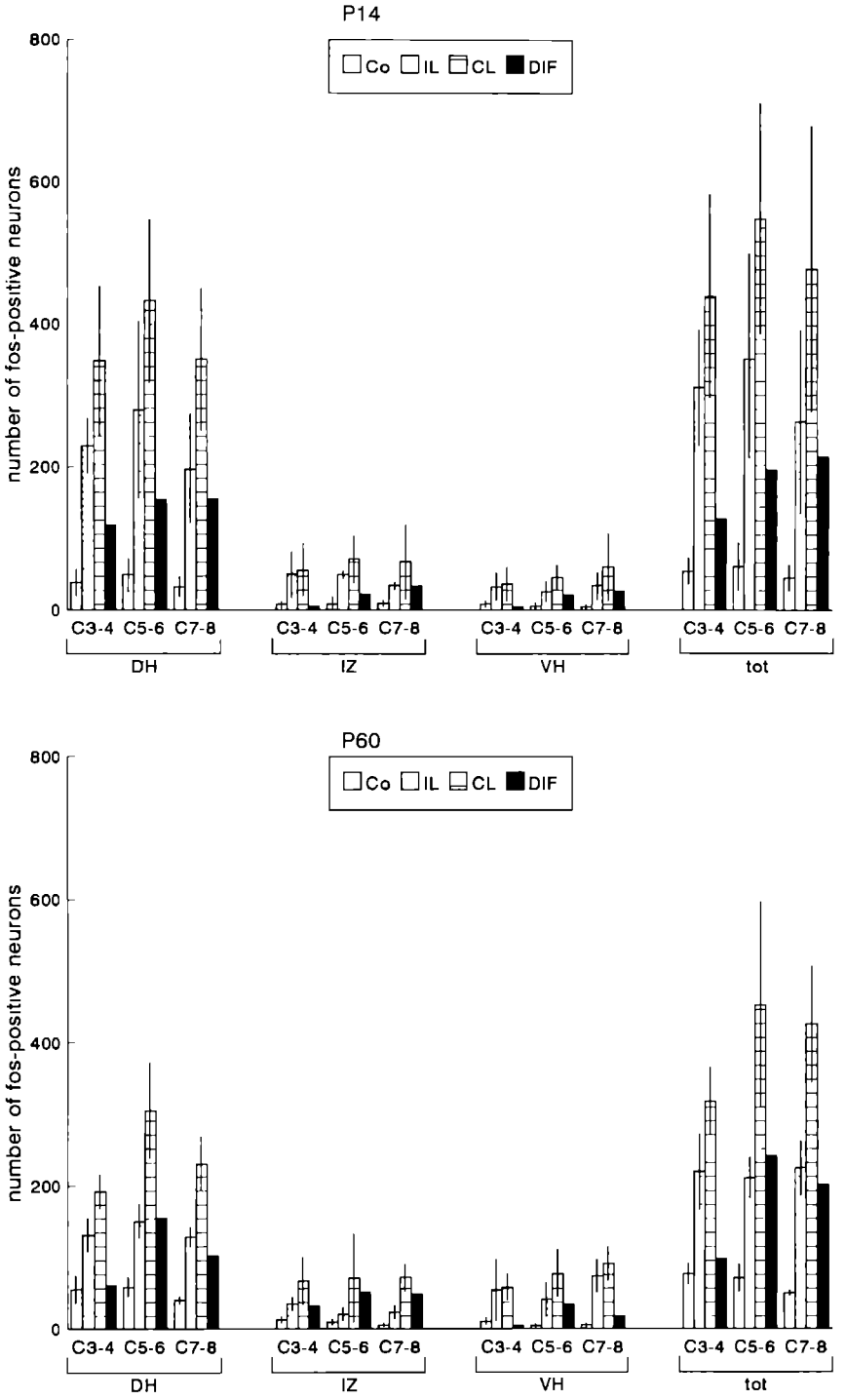

Fig. 4. Histogram showing the mean numbers and standard deviation of Fos-Li neurons in sham-operated (Co) and motor cortex kainate injected rats in the combined cervical spinal cord segments 3 and 4 (C3-4), C5-6, and C7-8 at P14 (top panel) and at P60 (bottom panel). For kainate injected animals a further differentiation is made for the ipsilateral (IL) and contralateral (CL) side. The spinal cord is subdivided in dorsal horn (DH), intermediate zone (IZ), and ventral horn (VH), total numbers (tot) are also shown. From this figure it can be concluded that kainate injections result in more neurons being labelled for c-fos, and that the increase is largest in the dorsal horn, in the combined C5-6, and on the contralateral side at both ages. It is further apparent that a significant decrease is noted in the number of Fos-Li nuclei from P14 to P60 after kainate stimulation of the cerebral motor cortex both ipsilaterally and contralaterally.

77 General discussion

As stated in the general introduction, this thesis centers round two questions

- What is the target of the corticospinal tract in the rat cervical spinal cord?
- What is the influence of this target on the outgrowing corticospinal tract?

With the experimental evidence provided in the preceding chapters these questions will be addressed in the following paragraphs

8.1. The corticospinal target in the adult rat

8 1 1 Cervical motoneurons

Since the corticospinal tract (CST) is involved in especially the voluntary digital flexion movements and because its influence is mediated via motoneurons (MNs) in the spinal cord, directly and/or indirectly via interneurons, in chapter 3 the MNs in the cervical spinal cord were investigated using retrograde labelling with cholera toxin subunit B conjugated to horseradish peroxidase (CTB-HRP) In a related paper (Dederen et al , 1994), it was shown that CTB-HRP is a potent neuroanatomical tracer combining high sensitivity and relatively simple histochemical staining Special attention was paid to the digital flexor *m flexor digitorum profundus*, a muscle involved in volitional finger movements As a reference, the digital extensor *m extensor digitorum communis*, an antagonist muscle involved in especially locomotion, was likewise examined Several differences between the two retrogradely labelled MN populations were found It was confirmed that the position of their somata in the transversel plane is different Both populations are located in cervical spinal cord segments 7 and 8 the extensor MNs dorsolaterally in the ventral horn, immediately adjacent to the white matter whereas the flexor MNs are positioned medially from extensor MNs In addition, it was established that flexor MNs have a dendritic component directed dorsomedially which is absent in extensor MNs (Fig 1) Since neurons receive input through both their somata and their dendrites it can be assumed that the two populations differ in their synaptic input

Since the CST terminal field in the rat cervical spinal cord is not very well documented, and the developing CST projection area described for other mammals differs from that in the rat, in chapters 4 and 7 1 the extension of the CST was studied using anterograde tract-tracing from the cortex with horseradish peroxidase (HRP) Labelled CST axons were found in most of the contralateral spinal gray, with the exception of the lateral part of the ventral and dorsal horn and of large parts of the ventral and lateral white matter Some regional quantitative differences were found Most fibres were located in the

medial part of the intermediate zone and their number progressively decreased towards the outer boundary of the projection area (Fig 1) It was further noted that the density and extension of CST axons progressively increased rostrocaudally from cervical spinal cord segments 3 to 8

In combining the findings on the dendritic fields of the two MN populations with the CST projection area encountered, as summarized in Fig 1, several conclusions can be drawn Firstly, flexor MN somata are located within the lightly labelled CST projection area whereas extensor MNs lie just outside the CST projection field Secondly, extensor MN dendrites overlap only with the lightly labelled CST projection area whereas flexor MN dendrites, and their dorsomedially extending dendrites in particular, also extend into the intermediately labelled CST projection field It thus seems reasonable to assume that
78 CST axons will have more direct synaptic contacts with flexor MNs than with extensor MNs

In chapter 5, the presence of direct corticomotoneuronal synaptic contacts was investigated using anterograde HRP labelling of the CST combined with retrograde CTB-HRP labelling of flexor MNs at the electron-microscopic level Double labelled cortico-motoneuronal synapses were found on dendrites both near the soma as well as further distally, as was expected from the overlap between the CST terminal field and flexor MN somata and dendrites These synapses were characterized by round vesicles, and are thus most likely excitatory (Peters et al , 1991) It is thus shown that flexor MNs in the caudal cervical spinal cord of the rat are a direct target for the CST As was noted in the introduction (chapter 2 2), especially the caudal extension of the CST into the spinal cord and the deepest layer in the spinal gray with CST terminations correlated best with the ability to perform skilled finger movements (reviewed by Heffner and Masterton, 1975, Kuypers, 1982) Based on this, mammals were subdivided into four groups with increasing CST extension into the spinal cord and increasing extension into the spinal gray, in correlation with increased digital skills The finding of direct cortico-motoneuronal synaptic contacts further justifies the classification of the rat into the third group of animals, since the second group lacks direct cortico-motoneuronal contacts (Alstermark and Sasaki, 1985, Fujito et al , 1991, Illert and Wiedemann, 1984), which are present in the third and fourth group (Asanuma et al , 1979, Cheney and Fetz, 1985, Elger et al , 1977, Fritz et al , 1985, reviewed by Porter, 1987, Porter and Lemon, 1993), whereas the fourth group has the ability to perform the precision grip (reviewed by Porter, 1987, Porter and Lemon, 1993) The presence of direct corticomotoneuronal synaptic contacts in the rat, both on dendrites and on the somata of flexor MNs, was suggested previously in a double-labelling study at the light-microscopic level (Liang et al , 1991) In addition, electrophysiological experiments revealed that the CST was able to elicit not only EPSPs but also action-potentials monosynaptically in MNs in the cervical spinal cord (Elger et al , 1977) but not in the lumbar spinal cord (Janzen et al , 1977) It is generally accepted that the closer to the soma a synapse is located, the stronger the influence of that synapse is upon the behaviour of a neuron It is, however, interesting to take note of the segmental cable theory, using computer simulations Wolf et al (1992) were able to demonstrate that synapses located far from the soma exerted strong influence on that particular neuron by means of internal amplification due to the electrical properties of the dendritic membrane

Electrophysiological experiments, however, also revealed synaptic contacts between the CST and extensor MNs (Elger et al , 1977), as was further substantiated in the present investigation by the overlap found of the CST terminal field with the extensor MN dendrites The existence of these direct contacts, however, remains to be established at the ultrastructural level It seems likely that if these contacts exist, different numbers of direct CST synapses will be found in the two MN populations analyzed, based on the difference in overlap between the CST and the flexor and extensor MN dendrites, respectively, as described above In order to obtain a better understanding of the discrepancy between the control of the CST upon the respective muscles further quantitative electron-microscopical research has to be carried out

8 1 2 Cervical interneurons

Although it was demonstrated in this thesis that the rat corticospinal tract (CST) projects directly onto flexor motoneurons (MNs) in the cervical spinal cord, part of the corticospinal influence will occur via interneurons (INs) In chapter 6, the INs innervating digital flexor MNs were investigated using retrograde transneuronal tract-tracing with pseudorabies virus (PRV) This virus has a high affinity for neuronal cells and its advantage over other transneuronally transporting tracers has been well established Taking into account the relatively short survival times used, it seems likely that most labelled INs encountered project monosynaptically upon the respective flexor MNs Labelled INs were found throughout the entire length of the cervical spinal cord with maximal numbers in these segments where the flexor MNs are located When the position of PRV labelled neurons is compared to the CST terminal field (Fig 2A), it is

apparent that the majority of their somata is located in the area which is moderately innervated by corticospinal fibres Furthermore, many INs are positioned in the area upon which numerous CST axons project Although the overlap is obvious, it remains to be established whether the CST indeed synapses upon these INs In future investigations, this could be determined at the electron-microscopical level using double labelling of the CST anterogradely with horseradish peroxidase (HRP) and the spinal INs retrogradely with PRV, respectively

In chapter 7 1, spinal INs involved in the corticospinal pathway were determined by transsynaptic labelling using c-fos immunohistochemistry after kainate stimulation of the sensorimotor cortex In comparing the results obtained with those after anterograde tract tracing with HRP (Fig 2B), numerous c-fos labelled INs were encountered within the labelled CST terminal field These INs were located in the intermediate zone of the spinal gray as well as in the dorsal part of the ventral horn Although it can not be ascertained with this technique, the assumption can be made that these INs are innervated monosynaptically by the CST

In Fig 2B, both the INs retrogradely labelled after PRV injections in the distal forelimb flexor muscle and those anterogradely labelled by using the c-fos technique after kainate injections into the sensorimotor cortex are depicted in a combined schematized drawing From this figure it is obvious that a great number of both the retrogradely labelled INs and the anterogradely labelled INs are positioned in the intermediate zone and dorsal part of the ventral horn, i e the area which receives strong CST innervation It thus can be concluded that these INs are likely candidates to mediate the transmission of cortical information to MNs The data in the literature on the INs intercalated in the cortico-motoneuronal pathway are scarce and predominantly originate from the cat and monkey The propriospinal neurons in cervical spinal cord segment 3-4 in the cat are perhaps the best studied and their role in the transmission of information from the cortex to the MNs in cervical spinal cord segment 6 to thoracic segment 1 is well established (Alstermark et al , 1981, 1990, 1991, Alstermark and Sasaki, 1985, Illert and Wiedemann, 1984, Petterson, 1990) The other populations of INs encountered in this thesis are however relatively unknown Cortical neurons diverge upon several subpopulations of spinal MNs and INs (Cheney and Fetz, 1985, Cheney et al , 1985, reviewed by Porter, 1987, Wise, 1993) This mechanism probably accounts for the coordination observed between several muscles during the performance of a particular volitional movement (Drew, 1993) It should be mentioned however, that the situation is probably much more complicated since many more afferent systems will innervate the same INs and MNs (reviewed e g by Kuypers, 1964, 1981, Pearson, 1993) Future detailed anatomical and electrophysiological experiments are needed to fully understand the neuronal pathways in the spinal cord and the influence of the CST upon INs and MNs in particular

8.2. Relation between the developing corticospinal tract and its target

Studies on developing fibre systems, such as the corticospinal, retinotectal, and neuromuscular projections, revealed that the developmental processes of fibre pathways in general can be conceived as three consecutive phases (reviewed by e g Goodman and Shatz, 1993, Kalb and Hockfield, 1992, Navarette and Vrbova, 1993, O'Leary et al , 1990) During the first phase pathway selection occurs axons emanating from their parent neurons traverse along their characteristic path through the surrounding substrate towards their target Then, as the developing axons reach their target, the correct target is selected and synaptic contacts are established Finally, the appropriateness of these connections is tested and aberrant synapses and fibres are eliminated in a process called fine-tuning In the following subsections of this chapter, these three phases of corticospinal tract development will be discussed in view of the results described in this thesis

8 2 1 Pathway selection

This phase of corticospinal tract (CST) development was not addressed in this thesis, since CST pathway selection was the subject of numerous previous studies (see for a recent review Stanfield, 1992) Besides, it can be assumed this process occurs relatively independent of the target for the following reasons Firstly, there is a large difference in location between the parent CST neurons in the cortex and the CST target in the spinal cord Secondly, the capability to respond to the same environmental cues appears to be initially present in all layer V cortical neurons, resulting in all cortical areas projecting to the spinal cord, including those neurons which later become part of the corticopontine projection (Joosten et al , 1987, Joosten and van Eden, 1989, O'Donoghue et al , 1993, O'Leary and Terashima, 1988, Uozumi et al , 1988, reviewed by Kalil, 1988, O'Leary et al , 1990, Stanfield, 1992) This is further substantiated by the fact that in young rats in which the pons is destroyed or misplaced by X-radiation, corticopontine axons are still able to find their correct pathway (reviewed by O'Leary et al , 1990) It can be deduced from the literature that

pathway selection rather depends on environmental cues emitted by glial cells, other non-neuronal substrates, midline cells, as well as other neuronal pathways In general, a combination of three mechanisms appears to occur differential adhesion, chemotropism by way of gradients of diffusible neurotrophic factors, and repulsion, which all cause the growth cone at the distal end of the outgrowing axon to make pathway choices Strong evidence was provided that the same three mechanisms also play a role in CST pathway selection Differential adhesion was reported by way of neural and/or glial cell adhesion molecules, among them L1, chemotropism as a mechanism was substantiated by glial cells producing neurotrophic factors, and a repulsive role was demonstrated for oligodendrocytes (Joosten, 1989, 1990, 1991, Joosten and Gribnau, 1989, Joosten et al , 1990, Schwab and Schnell, 1991, reviewed by Schwab, 1990) CST pathway selection thus appears to be a predestined process, according to intrinsic characteristics of all cortical neurons in conjunction with the substrate

In terms of regeneration after lesion of the CST in particular and other fibre pathways in general, it should be mentioned that regrowth past the lesion site is the first prerequisite for functional recovery However, as pathway selection occurs independent of the target, research should be focused on the intrinsic characteristics of outgrowing fibre bundles in conjunction with their immediate surroundings in the process of growth cone guidance Recently it was shown that when the inhibitory influence of oligodendrocytes is diminished by radiation or antibodies aimed at myelin, the capability of regrowth of the CST in the adult rat is enhanced (Schnell et al , 1994, Schwab and Schnell, 1991, reviewed by Schwab, 1990) However, since total regrowth was not established, not to mention synaptogenesis, it can be concluded that various factors and mechanisms play a role in concert, thus justifying a more holistic approach to the research of pathway regeneration

8 2 2 Target selection

The process of corticospinal tract (CST) target selection comprises the phase of corticospinal fibres exiting the dorsal funiculus and entering the spinal gray, then growing towards their target, and eventually ending with the formation of synaptic contacts with interneurons (INs) and motoneurons (MNs) In chapter 4 it was shown that although CST fibres reach the caudal cervical spinal segments at postnatal day 2 (P2), first outgrowth into the spinal gray only starts at P4 More fibres gradually enter and extend further into the spinal gray, reaching maximal numbers and extension at P10 (Fig 3) This process most likely depends on the target, hence more specificity than during pathway selection is to be expected It was found that cortical areas which in the adult do not project to the spinal cord, and temporarily extend into the spinal dorsal funiculus during development, do not grow out into the spinal gray (Joosten et al , 1987, Joosten and van Eden, 1989) This suggests that only a subpopulation of cortical pyramidal neurons is capable to respond to cues which induce outgrowth into the spinal grav

The difference of two days between the arrival of CST axons in the dorsal funiculus and the first outgrowth into the spinal gray is attributed to interstitial budding of axon collaterals (Gribnau et al , 1994, Joosten et al , 1994) and was also found in case of the corticopontine projection (O'Leary and Terashima, 1988) The underlying mechanisms to this waiting period are still unknown, however, the following hypothesis of mutual influence can be postulated Upon arrival in the dorsal funiculus, a diffusible factor may be emitted by the CST into the spinal gray This factor then might stimulate the target to produce another diffusible factor, which in turn induces the outgrowth of CST fibres into the spinal gray This hypothesis will be exemplified by means of the maturing MN populations in the caudal cervical spinal cord, as described in chapter 3 It should be kept in mind that in various parts of the spinal cord, the CST projects onto different targets, and that even in cervical spinal cord segment 7-8 additional targets exist Besides, it was shown that single CST axons also project to multiple targets in the spinal cord (Kuang and Kalil, 1990, 1994) When the developing CST and the maturation of its motoneuronal target are compared (Fig 3), several observations can be made The first CST fibres arrive in the dorsal funiculus in cervical spinal cord segments 7-8 at P2, at which age the flexor MN dendrites are still submaximal Flexor MN dendrites reach maximal numbers and extension at P4, and at this age the first outgrowth of CST axons into the spinal gray is found It remains to be determined to which extent these two developmental events are correlated Future *in vitro* experiments in our department (Gribnau et al , 1994) might reveal the exact nature of the interaction between the CST and its target, including the molecules which play a role in this process Using this technique, it was already demonstrated that spinal cord explants have a trophic influence on cortical explants (Joosten et al , 1991)

Anterograde tracing experiments in young rats further revealed that CST fibres originating in the more anterior part of the cortex preferentially grow out into the cervical spinal gray, whereas those originating in the more intermediate part grow out into the thoracic and lumbar spinal gray (Joosten et al , 1987) This further indicates the ability of CST axons

originating in different parts of the cortex to respond to specific cues emitted from the segmentally organized target (Kuang and Kalil, 1994) Hox genes, the vertebrate equivalents of the *Drosophila* homeotic genes, control the establishment of segmental identities during development (Kessel, 1993, Le Mouellic et al , 1992) Using retrograde tracing with CTB-HRP, we have shown that a null mutation of one particular Hox gene, i e *Hoxc-8*, altered the segmental organization of flexor and extensor MNs in the mouse (Tiret et al , submitted) The rostral boundary of both populations of MNs appeared to be shifted more rostrally in the mutant mice, and in addition they had assumed an altered position in the mediolateral plane It would be very interesting to test whether this genetically changed CST target is reflected in an altered corticospinal innervation of the flexor MNs In favour of this supposition is the fact that *Hoxc-8* mutant mice show large impairment of digital movements

It is apparent that if the CST target finding is mediated by the target, a complex but highly specific interaction of the CST and its respective targets must exist, which is still far from being understood Many more experiments are needed to establish the various targets *in vivo* and their influence upon CST outgrowth *in vitro* An additional complicating factor may be the fact that a certain target is not exclusively innervated by the CST, but also by other descending tracts as well as other neurons Although future research has to reveal whether these tracts may use similar mechanisms as the CST in target finding, research *in vivo* is even more complicated since these pathways develop largely prenatally in contradistinction to the CST

Once the CST axons have entered the spinal gray, further outgrowth occurs in all directions, reaching maximal extension at P10 (Fig 3), when the entire gray matter is covered It thus appears that CST fibres stop their outgrowth upon reaching the white matter, with the exception of the medial part of the lateral funiculus This might be due to the attainment of the outer border of the gray matter and thus the end of access to a positive factor and/or the extension up into the white matter and the impact of an inhibitory factor At P4 no overlap between corticospinal axons and flexor MN dendrites was found and thus no synaptic contacts, whereas at P7, when the overlap is apparent synaptic contacts are numerous (chapter 5) When maximal extension and numbers of CST fibres is reached and the synaptic contacts with the target are established, the phase of target selection is completed and the final stage, i e the fine-tuning, is entered

8 2 3 Fine-tuning

Studies on the developing retinotectal and neuromuscular projection revealed that synaptic contacts are established in a relatively aspecific manner, and only later on during development the connection is tested and if not correct is eliminated This results for instance in the motor endplate of the neuromuscular junction being innervated polyneuronally during development, whereas in the adult only one motoneuron (MN) innervates one motor endplate At a certain time during development redundant synapses and axon collaterals are eliminated This process of fine-tuning was shown to be dependent of the target it is assumed that axons compete for one and the same factor emitted by the target depending upon the activity of the latter (Antonini and Stryker, 1993, Bennett and Lavidis, 1984, Brown and Booth, 1983, Brown et al , 1976, Caroni and Becker, 1992, Harris and McCaig, 1984, Henderson et al , 1993, Lance-Jones, 1982, Oppenheim, 1991, Vanselow et al , 1990, Walton et al , 1992, reviewed by Goodman and Shatz, 1993, Kalb and Hockfield, 1992, Lichtman and Balice-Gordon, 1990, Lowrie and Vrbova, 1992, Navarette and Vrbova, 1993, Oppenheim, 1989)

In analogy to the above mentioned mechanism of fine-tuning, it can be postulated that corticospinal fibres initially form numerous connections with their targets during the target selection phase Then, after testing these contacts redundant synapses and axons are eliminated In this thesis, exuberant outgrowth of corticospinal axons during development was found (compare Figs 3 and 1) As was shown in chapter 4, at P10 maximal numbers and extension of corticospinal fibres in the spinal gray are reached, whereas later on during development aberrant fibres are eliminated In addition, MNs showed an increase in number and extension of their dendrites in the first postnatal week, and in the following weeks a decrease was noted (chapter 3, compare Figs 3 and 1) In chapter 7 2 the question was addressed whether transient connections are formed during development using the expression of the immediate early gene c-fos after kainate stimulation of the sensorimotor cortex A decrease of the area and the number of c-fos labelled interneurons was noted between P14 and P60 In this thesis no direct experimental evidence was presented concerning the last aspect of fine-tuning, namely the activity dependent emission of a target derived factor It should be mentioned however that although young rats in the first postnatal week show some motor behaviour, this principally is based on reflexes, and only from the end of the first postnatal week onwards the development of adult motor capacities in the rat become visible (Cazalets et al , 1990, Donatelle, 1977, Eilam and Golani, 1988, Westerga and Grams-

bergen, 1990, 1993). Only when the last developing fibre pathway, i.e. the corticospinal tract, starts to make synaptic contacts with its target, a wide range of initially immature motoric activities is displayed.

8.3. Concluding remarks

In the preceding sections, evidence was provided that the development of the corticospinal tract (CST) can be subdivided into three phases. During the first phase, the pathway is selected. This process is relatively independent of the target; it is the growth cone at the distal end of the elongating axon which makes pathway choices based on cues derived from the environment. During the second phase, the target is selected. After a waiting period of two days, CST axons form collaterals by interstitial budding as a reaction to a target derived factor. This process is characterized by more specificity than the process of pathway selection, however the axons entering the spinal gray overgrow their target. This phase ends with the formation of synaptic contacts with the target. During the third phase, the system is fine-tuned; redundant synapses and axons are eliminated through competition for a limited factor derived activity-dependently from the target.

In this thesis evidence was provided for the assumption that at least part of the development of the CST, as is the case in other long descending pathways in the central nervous system, depends upon the target. It is, however, apparent that the exact nature of the processes occurring during normal development of fibre bundles is still far from being fully understood. The redundancy of transient projections in the developing central nervous system probably accounts for the high degree of its plasticity to react to injuries. This capacity disappears at a certain time during maturation (Antonini and Stryker, 1993; Armand and Kably, 1993; Kalil and Skene, 1986; Kuang and Kalil, 1990; Martin and Xu, 1988; Merline and Kalil, 1990; reviewed by Kalil, 1988), along with changes of conditions found exclusively during development (Alcantara and Greenough, 1993; Alisky et al., 1992; Cabalka et al., 1990; Gorgels et al., 1987, 1989; Joosten, 1989; Joosten and Gribnau, 1989; Joosten et al., 1990; Kalil and Skene, 1986; Martı et al., 1987; McConnell et al., 1989; Smeyne et al., 1992; Villar et al., 1989; reviewed by Kalil, 1988). Attempts to increase the regenerative capacity of the adult central nervous system should be aimed at providing similar conditions as found during development, as appears to occur in the peripheral nervous system after a lesion (Borke et al., 1993; Brushart, 1993; Chiu et al., 1993; reviewed by de Felipe et al., 1993). This implicates that attention should not only be focused on the phase of pathway selection, but moreso on the stages of target finding. It is however also obvious that many experimental studies are still necessary to understand the mutually interrelated influence of the large diversity of cells and molecules upon developing fibres before a successful attempt can be made to achieve functional recovery after lesion in the central nervous system.

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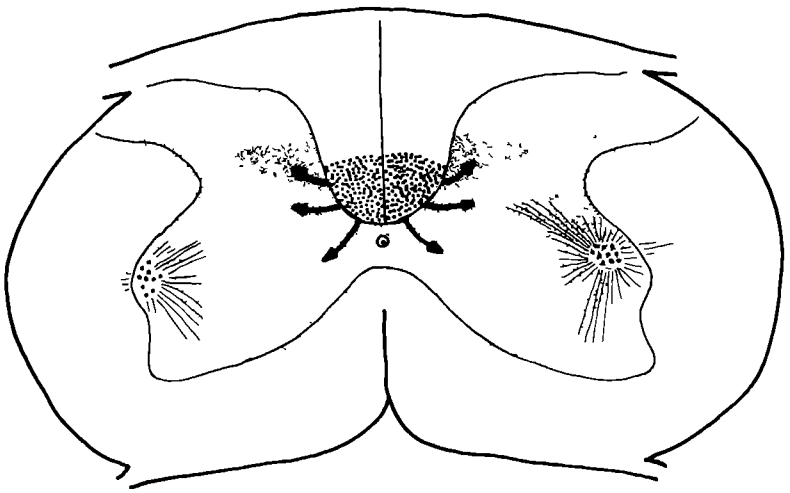

Fig. 1. Schematized drawing of cervical spinal cord segment 7 in the young adult rat CST labelling after HRP-gel implantation into the sensorimotor cortex was plotted in combination with the labelling observed in the respective MN populations after injection of CTB-HRP into the distal forearm flexor and extensor muscles. The left half of the figure depicts the extensor MNs and the right half the flexor MNs MN cell bodies are indicated by the large dots, their dendrites by the lines, the main CST bundle by the dots in the dorsal funiculus, and CST fibres in the spinal gray by the small dots The density of the latter reflects the difference in the amount of labelling. From this figure it can be concluded that the CST projection area covers the flexor MN somata and large parts of their dendritic fields and only part of the extensor MN dendrites.

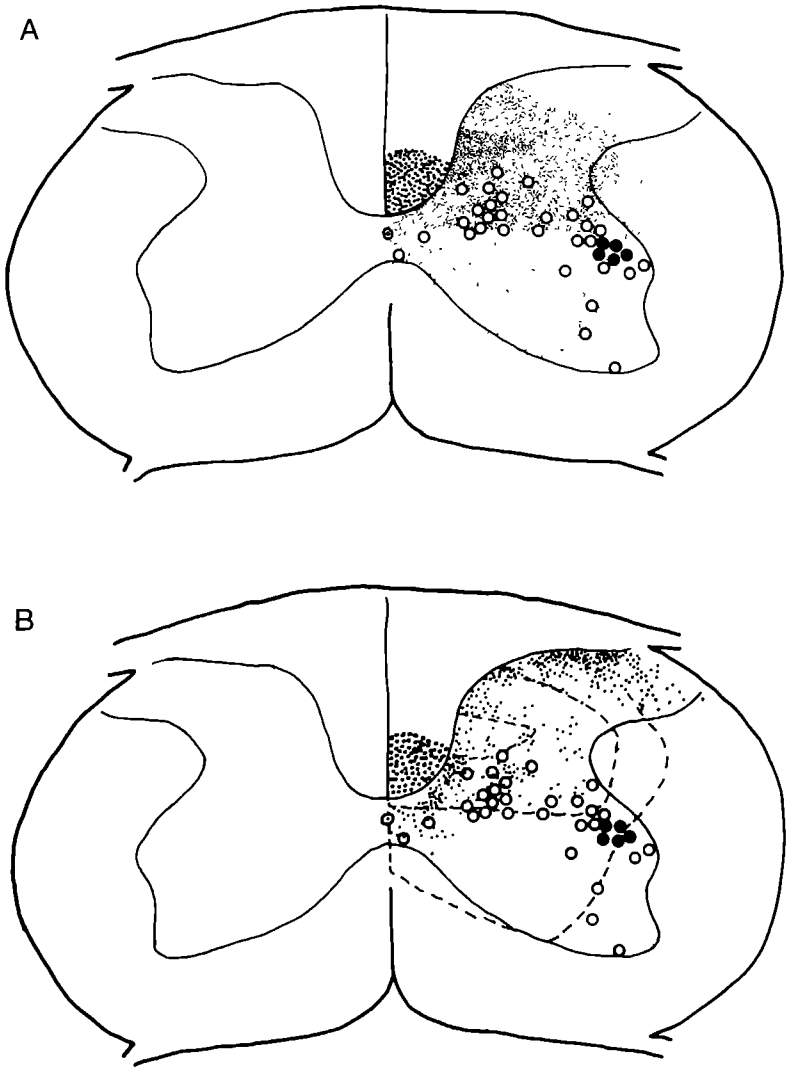

Fig. 2. Schematized drawings of cervical spinal cord segment 7 in the young adult rat. A. CST labelling after HRP-gel implantation into the sensorimotor cortex was plotted in combination with the neuronal labelling observed after injection of the transneuronally transporting PRV into the distal forearm flexor muscle. MN cell bodies are indicated by the filled circles, IN somata by the open circles, the main CST bundle by the dots in the dorsal funiculus, and CST fibres in the spinal gray by the small dots. The density of the latter reflects the difference in the amount of labelling. From this figure it can be concluded that INs projecting to distal flexor MNs are located in the area which receives CST input. The majority of these INs are confined to the area which is intermediately projected upon by the CST. B. The labelled c-fos positive INs after kainate injections into the motor cortex was plotted in combination with the labelled INs after PRV injections into the distal forearm flexor muscle. MN cell bodies are indicated by the filled circles, PRV labelled IN somata by the open circles, c-fos labelled INs by the small dots, the main CST bundle by the dots in the dorsal funiculus, and the areas of labelled CST fibres in the spinal gray (see Fig. 2A) by the dashed lines. From this figure it can be deduced that both populations partially overlap, and thus that the respective INs are likely candidates for the transmission of cortical output to MNs.

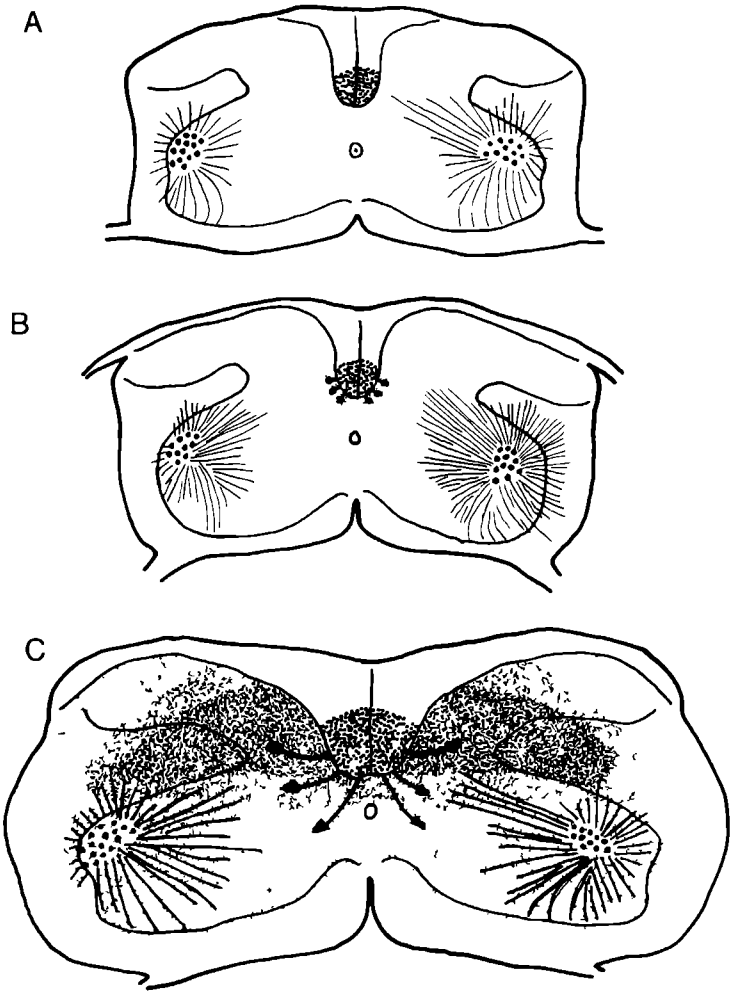

Fig 3 Schematized drawings of cervical spinal cord segment 7 at postnatal day 2 (A), 4 (B), and 10 (C) The labelling of the CST after HRP-gel implantation into the sensorimotor cortex was plotted in combination with the labelling observed in the respective MN populations after injection of CTB-HRP into the distal forearm flexor and extensor muscles The left half of the figure depicts the extensor MNs and the right half the flexor MNs MN cell bodies are indicated by the large dots, their dendrites by the lines, the main CST bundle by the dots in the dorsal funiculus, and CST fibres in the spinal gray by the small dots At P2, the first CST fibres arrive in the dorsal funiculus and the MN dendritic fields are submaximal At P4, first CST outgrowth into the spinal gray is found and especially flexor MN dendrites show maximal numbers and extension At P10, CST outgrowth into the spinal gray is maximal (compare to Fig 1), whereas the dendritic field already has started to decrease

SAMENVATTING

Eeuwenlang al is de mens geboeid door de vraag hoe nieuwe individuen van een soort gevormd worden Van het idee dat leven ontstaat uit een combinatie van de vier elementen water, lucht, vuur en aarde (Aristoteles, 4e eeuw voor Christus) kwam men met de toepassing van microscopen tot het inzicht dat een zaadcel al een compleet mini-individu bevatte dat alleen nog maar behoefde uit te groeien (Anthonie van Leeuwenhoek, 17e eeuw na Christus) Tegenstanders van deze laatste opvatting meenden dat niet de zaadcel, maar de eicel het mini-individu bevatte Sinds het midden van de vorige eeuw echter is algemeen geaccepteerd dat bij geslachtelijke voortplanting een eicel samensmelt met een zaadcel en dat hieruit een nieuw individu ontstaat Dit is het aandachtsveld van de embryologen, namelijk te bestuderen hoe uit deze ene bevruchte eicel een organisme ontstaat, bestaande uit miljarden en miljarden cellen die elk gekenmerkt worden door karakteristieke vormen, eigenschappen en functies

Hoofdstuk 1 bevat een inleiding op de algemene embryologie van gewervelde dieren, en zoogdieren in het bijzonder Aan de orde komt onder meer het ontstaan van de verschillende kiemlagen, het ectoderm, mesoderm en entoderm, de eerste specialisatie van het zich ontwikkelende embryo Door een subtiel samenspel tussen het ectoderm en het daaronder gelegen mesoderm ontstaat in de eerstgenoemde laag het neuro-ectoderm van waaruit het centrale zenuwstelsel gevormd wordt Het is het terrein van de neuro-embryologen de processen te begrijpen die leiden tot het ontstaan van dit zeer complexe orgaan, met zijn miljarden cellen (neuronen) die op hun beurt talloze verbindingen vormen met andere neuronen Behalve een fundamenteel wetenschappelijke waarde heeft dit type onderzoek ook nog een mogelijke toegepaste waarde terwijl gedurende de embryonale ontwikkeling neuronen in staat zijn uitlopers te vormen en correcte verbindingen te maken, is dit vermogen in volwassen neuronen in het centrale zenuwstelsel verdwenen Dit heeft tot gevolg dat wanneer er een beschadiging in het centrale zenuwstelsel plaatsvindt, er geen herstel optreedt Begrip van de embryonale ontwikkeling van het centrale zenuwstelsel kan er toe leiden inzicht te verkrijgen in de mechanismen die een rol spelen bij het tot stand komen van neuronale verbindingen Deze kennis kan vervolgens benut worden om de vraag te beantwoorden hoe beschadigde neuronen aangezet kunnen worden tot hernieuwde uitgroei en uiteindelijk regeneratie van zenuwbanen

In **hoofdstuk 2** wordt de corticospinale baan geintroduceerd Dit is een neuronale vezelverbinding die ontspringt in de schors (cortex) van de grote hersenen en die eindigt in het ruggemerg (medulla spinalis) De corticospinale baan speelt onder meer een rol in de controle van fijne, vrijwillige bewegingen van de vingers Deze vezelbundel wordt door neuro-embryologen vaak als onderzoeksmodel gebruikt, omdat hij bij knaagdieren pas na de geboorte uitgroeit in het ruggemerg Het is dus relatief eenvoudig experimenten uit te voeren zonder dat de embryo's in de baarmoeder gemanipuleerd hoeven te worden, met alle risico's van dien Onderzoek binnen de afdeling Anatomie en Embryologie was tot nu toe gericht op de processen die een rol spelen bij de uitgroei van de corticospinale baan van de rat in de witte stof (het deel van het ruggemerg waar de vezels zich bevinden) van het ruggemerg Echter, de uitgroei van de corticospinale vezels in de grijze stof (dat deel van het ruggemerg waar de neuronen zich bevinden) en de contactvorming met andere neuronen is tot nu nog nauwelijks onderzocht Dit is dan ook het onderwerp van dit proefschrift

Aangezien de corticospinale baan betrokken is bij de besturing van fijne, vrijwillige bewegingen van de vingers, en de buiging (flexie) van de vingers in het bijzonder, werden in **hoofdstuk 3** de neuronen onderzocht die de respectievelijke spieren, betrokken bij deze bewegingen, aansturen de flexor motoneuronen Deze cellen werden gelabeld met behulp van de merkstof cholera toxine, subunit B Dit is een molecuul dat na injectie in de spier door de vezels van de motoneuronen naar hun cellichaam in het ruggemerg wordt getransporteerd Een bijkomend voordeel van deze stof is dat niet alleen het cellichaam gelabeld wordt, maar ook de uitlopers van dat cellichaam waarmee vezels van andere cellen contact kunnen maken de dendrieten Uit het onderzoek blijkt nu dat de cellichamen zelf gedurende de postnatale ontwikkeling geen veranderingen ondergaan in aantal en positie Hun dendrieten echter nemen gedurende de

eerste postnatale week toe in aantal en lengte, om vervolgens weer geleidelijk af te nemen

In **hoofdstuk 4** wordt het onderzoek van de uitgroei van de corticospinale baan in de grijze stof beschreven In de literatuur bestaat er een discrepantie wat betreft deze uitgroei in de rat en andere diersoorten De corticospinale vezels werden gelabeld met behulp van mierikswortel peroxidase Deze stof werd, ingebed in een gel-substantie, geimplanteerd in de schors van de grote hersenen Na door neuronen opgenomen te zijn wordt dit molecuul getransporteerd naar de vezels en hun uiteinden De aandacht was gericht op dat deel van het ruggemerg waar de flexor motoneuronen gelocaliseerd zijn zoals beschreven in hoofdstuk 3, namelijk de cervicale segmenten 7 en 8 Corticospinale vezels bereiken de witte stof op dit niveau op postnatale dag 2 Vervolgens vindt na 2 dagen de eerste uitgroei in de grijze stof plaats Tot en
90 met postnatale dag 10 worden steeds meer corticospinale vezels aangetroffen, die ook steeds verder in de grijze stof reiken Nog later verdwijnen er weer vezels en reiken de definitieve uitlopers ook minder ver in de grijze stof

Aangezien uit de literatuur bekend is dat het doel, dat zijn de cellen waar neuronen contact mee maken, de uitgroei van vezelbundels beinvloedt, werd in **hoofdstuk 5** bepaald of de motoneuronen die in hoofdstuk 3 onderzocht werden een doel vormen voor corticospinale vezels Daartoe werden flexor motoneuronen gelabeld met cholera toxine subunit B en de cortex neuronen en de corticospinale vezels met mierikswortel peroxidase Contacten (synapsen) tussen beide populaties neuronen werden gezocht met behulp van een electronenmicroscoop Directe verbindingen werden voor het eerst gevonden op postnatale dag 7 en ook in volwassen ratten werden deze contacten aangetroffen, hetgeen bevestigt dat flexor motoneuronen een direct doel vormen voor corticospinale vezels

In het ruggemerg bevinden zich nog meer populaties neuronen die, hetzij direct als doel, hetzij indirect, een rol kunnen spelen bij de uitgroei van de corticospinale baan Allereerst werden in **hoofdstuk 6** de neuronen in het ruggemerg in kaart gebracht die contacten hebben met flexor motoneuronen. Deze zogenaamde interneuronen werden gelabeld met behulp van het pseudorabies virus Dit virus werd in de spieren geinjecteerd en labelt in eerste instantie de flexor motoneuronen Vervolgens wordt het virus getransporteerd naar interneuronen via de verbindingen tussen beide populaties Op grond van de gevonden localisatie van de gelabelde interneuronen op diverse postnatale leeftijden kon geconcludeerd worden dat de positie ervan gedurende de postnatale ontwikkeling constant blijft Het is echter zeer wel mogelijk dat het aantal interneuronen, hun dendrieten en/of het aantal contacten met motoneuronen wel onderhevig is aan veranderingen, hetgeen nader onderzocht zou moeten worden

In **hoofdstuk 7** worden de interneuronen beschreven die verbindingen hebben met de corticospinale baan Om deze aan te tonen werd de schors van de grote hersenen gestimuleerd met kainezuur Dit heeft tot gevolg dat de neuronen die verbonden zijn met de schors, inclusief de interneuronen in het ruggemerg, op hun beurt ook sterk gestimuleerd worden Als reactie op deze sterke prikkeling worden onder andere de zogenaamde 'immediate early' genen in deze neuronen tot expressie gebracht Het produkt van deze genen kan vervolgens met behulp van specifieke antilichamen aangetoond worden In **hoofdstuk 7.1** wordt allereerst een vergelijking gemaakt in volwassen ratten tussen de expressie van zo'n 'immediate early' gen, namelijk c-fos, na stimulatie van de schors met kainezuur enerzijds en de labelling van de corticospinale vezels die gevonden wordt nadat mierikswortel peroxidase-gels geimplanteerd zijn in de schors anderzijds Het blijkt dat met name een bepaalde populatie c-fos gelabelde interneuronen in het ruggemerg wat betreft hun localisatie een behoorlijke overeenkomst vertoont met de positie van de gelabelde corticospinale vezels In **hoofdstuk 7.2** wordt vervolgens de vraag beantwoord of corticospinale vezels, die tijdelijk een uitgroei in de grijze stof van het ruggemerg vertonen, ook tijdelijke contacten aangaan met hun doel Hiertoe werd de c-fos expressie na stimulatie van de schors met kainezuur in ratten van 14 dagen oud vergeleken met die in volwassen ratten Op grond van het gevonden grotere aantal c-fos gelabelde interneuronen en hun positie kan inderdaad geconcludeerd worden dat gedurende de ontwikkeling contacten van tijdelijke aard bestaan tussen de corticospinale baan en het doel, in dit geval de interneuronen

In **hoofdstuk 8** tenslotte worden de resultaten zoals beschreven in de verschillende hoofdstukken van dit proefschrift met elkaar in verband gebracht en gerelateerd aan de gegevens uit de internationale literatuur In grote lijnen kan de ontwikkeling van de corticospinale baan in drie fases worden onderverdeeld Tijdens de eerste fase, die aangeduid kan worden als de selectie van het te volgen pad, groeit de baan door de hersenen naar het ruggemerg toe en vervolgens in de lengterichting door de witte stof van het ruggemerg heen Het uiteinde van een uitgroeiende vezel wordt gekenmerkt door een verdikking de groeiconus In de groeiconus worden beslissingen genomen over het te volgen traject mede op basis van informatie uit de omgeving Dit proces treedt min of meer onafhankelijk op van het doel Tijdens de twee-

de fase wordt het doel geselecteerd. Na een wachttijd van twee dagen vormen de vezels zijtakken, de zogenaamde collateralen, die beginnen uit te groeien in de grijze stof. Gedurende deze twee dagen wacht de corticospinale baan waarschijnlijk op een factor die afgegeven wordt door het doel, mogelijk als reactie op de aanwezigheid van de corticospinale baan. Hoewel in deze fase al meer specificiteit wordt gevonden dan tijdens de eerste fase is er nog sprake van een grote mate van overvloed. Talrijke vezels groeien namelijk hun doel voorbij om vervolgens later geëlimineerd te worden. De tweede fase eindigt dan met de vorming van vele contacten met het doel. Tijdens de derde fase treedt een fijn-afstemming van het systeem op. Overbodige contacten en vezels worden geëlimineerd waarschijnlijk doordat de vezels met elkaar concurreren om een specifieke factor die slechts in beperkte mate aanwezig is. Verondersteld wordt dat deze factor afhankelijk van de activiteit door het doel wordt afgescheiden, met andere woorden, de neuronen in de schors testen zelf de synapsen door deze te prikkelen. Het is in dit verband vermeldenswaard dat op het moment dat de ontwikkeling van de corticospinale baan de derde fase ingaat, de motoriek van de ratten drastisch begint te veranderen en sterk gaat lijken op het volwassen gedrag

In dit proefschrift zijn aanwijzingen gevonden dat het doel van de corticospinale baan de uitgroei ervan beinvloedt. De exacte mechanismen die daarbij een rol spelen zijn echter nog onbekend. Wel is duidelijk dat gedurende de ontwikkeling bepaalde kenmerken verdwijnen of veranderen en dat daarna in het volwassen systeem het vermogen tot aanpassen aan beschadigingen is verdwenen. Onderzoek met het oog op het bevorderen van het regeneratieve vermogen van het centrale zenuwstelsel moet dan ook gericht zijn op het opnieuw creeren van dezelfde gunstige eigenschappen zoals die gedurende de ontwikkeling aanwezig zijn. Hierbij moet dan ook de aandacht gericht zijn op alle drie de fasen van de ontwikkeling, dus niet alleen op de selectie van het pad, maar zeker ook op selectie van het doel en de fijn-afstemming van het systeem.

Uit dit onderzoek is vooral duidelijk geworden dat nog veel research nodig is om de complexe invloed van de verschillende cellen en factoren op de uitgroeiende vezels te begrijpen. Pas dan kan een succesvolle poging ondernomen worden om functioneel herstel te bewerkstelligen na een beschadiging van het centrale zenuwstelsel.

Dankwoord

Hoewel alleen mijn naam op de kaft prijkt, zijn er natuurlijk veel meer mensen direct of indirect betrokken geweest bij de totstandkoming van dit proefschrift En het is hier dat ik hen oprecht mag bedanken

Op de eerste plaats dank ik mijn dagelijks begeleidster voor de kans die zij me geboden heeft dit onderzoek uit te voeren Yessie, je liet me de vrijheid mijn eigen richting te kiezen terwijl je subtiel bijstuurde om me toch op de juiste koers te houden Jouw kritische opmerkingen en kennis van de literatuur hebben er mede toe geleid dat het proefschrift zijn huidige vorm heeft gekregen Ook het andere lid van het neuro-embryologie team is van onschatbare waarde geweest Jos, jouw inzicht en eigenwijsheid (in de meest gunstige zin) maakten dat ik me bewust bleef van de noodzaak mijn keuzes te kunnen beargumenteren Verder heb ik jouw flexibiliteit enorm gewaardeerd als ik weer eens te veel gepland had, hielp je me toch uit de brand, ook al was de 'fluit' gegaan

Mijn promotor verdient een aparte paragraaf Prof Nieuwenhuys, uw kritische opmerkingen tijdens met name de laatste fase van het onderzoek hebben voor de spreekwoordelijke puntjes op de i gezorgd De snelheid waarmee U de manuscripten, voorzien van suggesties en de zwakke punten rakend, weer wist te retourneren hebben mij telkens opnieuw verbaasd

Ine, jou dank ik voor de hulp bij de fos-experimenten, maar nog veel meer voor de oprechte belangstelling voor mijn werk en welzijn Theo en Henk, jullie hebben het verblijf in het tijdelijke onderkomen (het aquarium) wat plezieriger gemaakt En verder dank ik hier natuurlijk alle andere (oud-)medewerkers en collega-promovendi van de afdeling Anatomie en Embryologie van wie de bijdrage misschien minder concreet is geweest, maar die erv oor gezorgd hebben dat ik af en toe ook eens de blik op iets anders richtte dan alleen maar mijn werk

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Het laatste deel van dit dankwoord reserveer ik voor de mensen in mijn persoonlijke omgeving Mijn ouders dank ik voor het feit dat zij me altijd gestimuleerd en gesteund hebben Papa, ik had graag gewild dat je de voltooiing nog meegemaakt had, wat zou je trots geweest zijn Mama, ik bedank jou speciaal dat je toch nog de kracht gevonden hebt om verder te gaan Ik noem hier ook mijn zoon, Rutger, die me in al zijn onschuld de relativiteit van dit proefschrift getoond heeft En op de laatste plaats bedank ik jou, Stephanie, voor het begrip en geduld zodat ik mijn proefschrift op de eerste plaats kon stellen Ik weet dat het niet altijd gemakkelijk is geweest, maar ik hoop dat het weer wat rustiger wordt en ik wat meer tijd voor ons heb

Curriculum vitae

De auteur van dit proefschrift werd 6 juli 1965 geboren te Heer. Nadat de lagere school 'de Joppenhof' te Heer met goed gevolg werd doorlopen, werd in 1983 het atheneum-B diploma behaald aan de 'scholengemeenschap Jeanne d'Arc' te Maastricht. In datzelfde jaar begon hij met de studie biologie aan de Katholieke Universiteit Nijmegen, na uitgeloot te zijn voor de studie diergeneeskunde. In 1984 behaalde hij het propaedeutisch examen biologie en koos ervoor deze studie te voltooien. In 1990 werd het doctoraal examen afgelegd, dat de hoofdvakken Dierfysiologie (prof. dr. Wendelaar-Bonga) en Psychoneurofarmacologie (prof. dr. Cools) en de bijvakstage Celbiologie en Histologie (dr. Eling) omvatte.

Van 1990 tot 1994 was de auteur werkzaam als Assistent in Opleiding bij de vakgroep Anatomie en Embryologie, Faculteit der Medische Wetenschappen, Katholieke Universiteit Nijmegen, onderdeel van het 'Nijmegen Institute for Neurosciences' en de onderzoekschool 'Pathofysiologie van het Zenuwstelsel' (Katholieke Universiteit Nijmegen, Rijksuniversiteit Utrecht en Landbouwuniversiteit Wageningen). In dit laboratorium werd onder de verantwoordelijkheid van dr. A. Gribnau en onder supervisie van prof. dr. R. Nieuwenhuys het onderzoek, onder de titel 'Ontwikkeling van structuren betrokken bij de besturing van pootbewegingen bij de rat', uitgevoerd waarvan de resultaten beschreven worden in dit proefschrift. Hier assisteerde hij ook bij het embryologie-onderwijs aan studenten geneeskunde en gezondheidswetenschappen.

Momenteel is de auteur werkzaam als bioloog in het *In Vitro* Fertilisatie Laboratorium van het Sophia Ziekenhuis te Zwolle.

Hij deelt het dagelijks leven met vriendin Stephanie van Stokkum en zoon Rutger.

Colofon

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Layout en druk
Thoben Offset Nijmegen

Stellingen behorende bij het proefschrift

Development of the rat corticospinal tract

Target finding and fine-tuning
in the cervical spinal cord

I

De corticospinale baan bij de rat heeft directe contacten met motoneuronen
Dit proefschrift

II

Regeneratie van zenuwbanen is meer dan alleen hernieuwde vezeluitgroei, om uiteindelijk functioneel herstel te verkrijgen zijn 'target-selection' en 'fine-tuning' minstens even belangrijk
Dit proefschrift

III

De embryonale ontwikkeling wordt gekenmerkt door een overvloedige aanmaak, vervolgens wordt de overtollige structuren onder invloed van de omgeving geëlimineerd

IV

De vinding van onder andere *Bennet et al (J Comp Neurol, 218, 1983, 351-363), Iwamoto et al (Neurosci Lett, 20, 1980, 25-30), Nurcombe et al (Neurosci Lett, 27, 1981, 249-254), Rootman et al (J Comp Neurol, 199, 1981, 17-27)*, en *Tada et al (Exp Brain Res, 35, 1979, 287-293, Exp Neurol, 65, 1979, 301-314)*, dat HRP gelabelde motoneuronen projecterend naar een ledemaatspier over meer dan twee ruggemergsegmenten gevonden worden, is onjuist

V

Het feit dat adviezen van consultatie-bureaus aan ouders van zuigelingen een sterke regio-afhankelijke variabiliteit vertonen doet vermoeden dat de meeste van deze adviezen slechts op meningen en niet op objectieve kennis berusten

VI

Zolang universiteiten beloond worden voor elke afgestudeerde, is deze instellingen er alles aan gelegen zoveel mogelijk studenten de eindstreep te doen halen, hetgeen normverlaging in de hand werkt.

VII

Het afdoen van uitspraken, ook al zijn deze ongenuanceerd, met borreltafelpraat is pedant.

VIII

Met milieubeleid is niet uitsluitend de natuur, doch vooral ook de mens zelf gediend.

IX

Promoveren heeft veel van topsport. Alleen krijgen sporters na een topprestatie een bonus, promovendi het ontslag.

X

Experimenten aan embryo's terwille van het verbeteren van IVF-technieken en pré-implantatie diagnostiek zijn toelaatbaar.

MAX CURFS, 21 september 1995